THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: CALLIOPI HAVELE

TITLE OF THESIS: IMMUNOLOGICAL TOLERANCE AND AUTOIMMUNITY IN EMBRYONICALLY-GENERATED AVIAN HAEMATOPOIETIC CHIMAERAS

DEGREE FOR WHICH THESIS WAS PRESENTED: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: 1980

THE UNIVERSITY OF ALBERTA

IMMUNOLOGICAL TOLERANCE AND AUTOIMMUNITY IN EMBRYONICALLY-GENERATED AVIAN HAEMATOPOIETIC CHIMAERAS

by

© CALLIOPI HAVELE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF IMMUNOLOGY

EDMONTON, ALBERTA

SPRING, 1980

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled IMMUNOLOGICAL TOLERANCE AND AUTOIMMUNITY IN EMBRYONICALLY-GENERATED AVIAN HAEMATOPOEITIC CHIMAERAS submitted by CALLIOPI HAVELE in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in IMMUNOLOGY

DEDICATION

To my parents
for their continuous moral support
during the years of my studies.

ABSTRACT

Avian haematopoietic chimaeras have been produced at 4 different stages of ontogenic development between the two strains of chicken SC and FP. All of the chimaeras produced by parabiosis at day 12 of embryogenesis and the majority (83%) of the ones produced at day 15 by intravenous injection of 15 day old allogeneic stem cells of spleen origin, remain healthy, chimaeric and specifically tolerant throughout a long examination period. Tolerance was observed at both the cell-mediated and humoral level and was demonstrable at the time of hatching. There was no evidence that the tolerant state was mediated by active suppression. These birds appear to be tolerant in a way that is analogous to the unresponsiveness displayed by the immune system to self antigens. Chimaeras generated at day 17 of embryogenesis show specific unresponsiveness at the cell-mediated level but produce specific anti-donor strain antibodies which were detectable in their sera as early as the third week after hatching. These chimaeras and a minority (17%) of the chimaeras generated at day 15 of embryogenesis developed severe antibody-mediated autoimmune haemolytic anaemia after the fifth month of age. They succumbed to massive bursal and other lymphoid tumours by the tenth month of age. The immunological and pathological characteristics of these birds appear to reflect an autoimmune state rather than one of tolerance. Chimaeras generated at day 21 of ontogenic development displayed

normal levels of GVH reactivity. These birds were able to eliminate the chimaeric state and remained healthy until deliberately terminated. Cell-mediated immunocompetence, as measured by GVH reactivity, can first be detected at hatching but may actually arise at low levels even earlier. Chimaeras generated at hatching behaved qualitatively as adults, in that they were able to mount an effective cell-mediated immune response and rejected the foreign tissue.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Drs. Michael Longenecker and Thomas Wegmann, for their support and help during the course of this work. I am grateful to Dr. Linda Pilarski for her encouragement and for the many discussions that stimulated my interest in the field of cellular immunology. I would like to express my gratitude to Dr. F. Pazderka for her willingness to introduce me to some of the essential techniques used in this work. I thank my colleague Dr. Gordon Keller for the successful and enjoyable collaboration through the demanding years of our graduate studies. Finally, I would like to express my appreciation to Ms. Louiza Gordon for her efforts and expert typing during the preparation of this thesis.

LIST OF ABBREVIATIONS

aHGG:	Aggregated human gamma globulin
B Cells:	Bursa and/or Bone marrow derived lymphocytes
BSA:	Bovine serum albumin
CAM:	Chorioallantoic membrane
CAM-POCKS:	Chorioallantoic membrane pocks
d-GL:	Dextro-polyglutamic acid and lysine
d HGG:	Deaggregated human gamma globulin
DNP:	Dinitrophenyl group
DNP-Lys:	Dinitrophenyl lysine
DTH:	Delayed-type-hypersensitivity
Ea:	Egg albumin
EGG:	Equine gamma globulin
FP*:	FP birds chimaeric with SC red cells
GVH:	Graft-versus-host
HGG:	Human gamma globulin
HSA:	Human serum albumin
Hy:	Hemocyanin
I.P.:	Intraperitoneally
I.V.:	Intravenously
KLH:	Keyhole-limpet hemocyanin
Lys:	Lysine
LPS:	Lipopolysaccharide
LWC:	Large white colonies
MHC:	Major histocompatibility complex

MGG:	Mouse gamma globulin
MLC:	Mixed lymphocyte culture
MLR:	Mixed lymphocyte reaction
OVA:	Ovalbumin
PBC:	Peripheral blood lymphocytes
PFC:	Plaque-forming cell
PHA:	Phytohemagglutinin
P:	Pocks
RBC:	Red blood cells
SC*	SC birds chimaeric with FP red cells
SRBC:	Sheep red blood cells
SWC:	Small white colonies
T Cells:	Thymus derived lymphocytes

TABLE OF CONTENTS

page

page

LIST OF TABLES

page

LIST OF FIGURES

I. Introduction

1. Historical Introduction

The main purpose of the work presented in this thesis is to obtain and analyze an experimental model of immunological unresponsiveness analogous to that displayed by the immune system towards self components. In this review of the literature I shall describe some of the many experimental systems that have been developed with a similar aim in mind. I would like to give a historical description of how certain chance observations led to the recognition of self-nonself discrimination as a central problem in immunology. I shall also describe how these observations suggested experimental approaches towards solving this problem.

In 1939 Erick Traub summarized his observations and experimental results in which congenital infection of a mouse colony with lymphocytic choriomeningitis virus, over a period of four years, gave rise to a long lasting asymptomatic infection. In 1945 R.D. Owen made the discovery that bovine dizygotic twins regularly contained a mixture of two types of erythrocyte which usually persisted for life.

These two independent experimental observations and particularly the fact that in the bovine chimaeras the genetically "wrong" cells persist without eliciting an

immune response but appear to be tolerated as self cells, led Burnet and Fenner in 1949 to introduce the concept that there is an acquired ability of the immune system to discriminate between self and non-self antigens. At that time, in their "indirect template theory", they predicted that the body's components are immunologically innert and that the equivalent state of tolerance to foreign antigens could be experimentally induced if these antigens are introduced at an appropriate stage of embryonic life. To account for the non antigenicity of the body's self antigens they postulated that they carry a "self marker" which is detected by a "recognition unit" acting at some point in the process leading to antibody production. Presumably the introduction of foreign antigens early in development resulted in their acquisition of the self-marker. It was postulated that the interaction of the "recognition unit" with the "selfmarker" prevented the immune system from responding. Although this theory had already postulated the acquired nature of immunological tolerance, it remained essentially instructional in its attempts to explain the production of antibody diversity.

In 1955, Jerne published his theory of "natural selection" where the concept of differentiation of self from nonself found a less abstract explanation than that of "self marker" and "recognition unit". He postulated that naturally occuring gamma globulin molecules are true antibodies, and that there are such antibodies specific for all foreign

antigens. He further suggested that autoantibodies are eliminated during fetal life and are not available later for selection and multiplication. Jerne discarded altogether the requirement of antigenic instruction for antibody production which had been the central dogma in all theories which dominated the field up to that period (Brein. and Haurowitz, 1930; Pauling and Cambell, 1942) with the exception of Ehrlich's side chain theory. Nevertheless Jerne's ideas retained instructional aspects at the molecular level as they failed to incorporate the already known facts on the mechanism of protein synthesis. He postulated that natural serum antibody, after its union with antigen, is brought to a random antibody producing cell. This particular cell was postulated to be stimulated to produce exact copies of the original natural antibody. In other words, he still envisaged that a protein can instruct a random cell to produce that protein.

Two years later, in 1957, Talmage put together Jerne's "natural selection theory" and Ehrlich's concept of selection of antibodies on a cell surface. In his pioneering proposal in 1900, Ehrlich assumed that all foreign antigens, especially bacterial toxins, damage the body's cells by combining with "side chains" on cell surfaces. The union was assumed to be specific and irreversible and the damage to the cell could only be overcome by casting off the blocked "side chain" and replacing it with a new one. The theory clearly implied the existence of cellular patterns

complementary to all possible antigenic determinants. Talmage pointed out that Jerne's concept of "natural selection" could find a more satisfactory form if antigenic selection occurs as a result of natural affinity between antigen and cellular receptors as Ehrlich had originally proposed. In that way the information for specific antibody production would not be carried to the cell from the outside but would be an intrinsic part of the cellular unit that is responsible for antibody formation. The information would be contained in the hereditary material already known to be the nucleic acids. According to Talmage, the process of natural selection requires the multiplication of a few cells out of a diverse population. He speculated that "one of the multiplying units in the antibody response is the cell itself. Only those cells are selected for multiplication whose synthesized product has affinity for the antigen injected". Talmage's proposal essentially included all the basic elements of a clonal selection principle as the basis of the immune response. Unfortunately, he did not elaborate his views further.

In 1957 Burnet also prepared an initial theoretical proposal that incorporated the main features of Talmage's hypothesis and in addition provided an explanation for two sets of phenomena, an understanding of which he had, since 1949, considered to be essential features in the process of antibody formation. These two phenomena were the non-antigenicity of the body's components and the existence of

immunological "memory". Burnet's views were extensively stated in 1959 in his "Clonal Selection theory of Immunity". The theory postulated "a randomization of pattern amongst differentiating lymphoid cells in embryonic life, so that each lymphoid cell in the body carried one immunological pattern expressed either as a receptor or as the specificity of antibody produced by the cell or its descendants. Subject to the possibility of mutation the pattern was transmitted by somatic inheritance to all descendants giving rise to large numbers of clones of cells, each with a distinct immunological specificity". This theory introduced the basic concept of the immunologically competent cell, a cell which is susceptible to specific stimulation on contact with the appropriate antigenic determinant. This interaction could lead a) to destruction of the cell if physiologically immature, b) to proliferation without changing its character to what is called a memory cell, and c) to proliferation and antibody production as a clone of plasma cells.

The literature of experimental embryology is rich with examples of embryos tolerating grafts of foreign origin. Their permanent acceptance into adult life was perhaps first demonstrated in 1929 when Danforth and Foster exchanged skin grafts between different strains of newly hatched chicks. They made the remarkable observation that about 10% of the transplanted grafts were accepted permanently. They concluded that the host's environment (e.g. hormones) could not influence the color of the donor skin, but they failed

to make any immunological inference.

In 1952 Cannon and Longmire, studying methods to improve graft survival between different individuals, essentially repeated Danforth's experiments with similar results. They went further to demonstrate the effect of the age of the recipient on graft survival. They found that 5 - 10% of the newly hatched grafted chicks retained the donor skin permanently. The graft survival dropped to 1% when the graft was implanted on the 5th day post hatching and none survived when implanted on day 14.

Although the above observations constitute the first experimental demonstration of transplantation tolerance induced in immunologically immature animals, the immunological basis of the phenomenon and its acquired and specific nature were first demonstrated in 1953 by the classical experiments of Billingham, Brent and Medawar in both mice and chickens. Pregnant CBA mice were injected in utero with a variety of lymphoid tissues from adult mice of strain A. Eight weeks later the litters were grafted with strain A skin and over 60% of the animals studied accepted the grafts permanently. The same mice were able to mount a vigorous rejection of AU strain skin grafts. It seems that the apparent lack of GVH reactions in these mice was in part due to the low number of adult lymphoid cells injected. The inoculum given was a mixture of adult tissues including testis, kidney and spleen. Another factor contributing to

the lack of a fatal GVH reaction might have been the limited genetic disparity between CBA and A strains of mice in the H-2 region. Although their stock of Rhode Island and White Leghorn chickens was non inbred, they also succeeded in inducing transplantation tolerance in chickens. Tolerance was achieved by injecting 0.2 ml of embryonic blood from one member of one breed of chicken into 12-day-old embryos of the other breed. Tolerance was highly specific in that donor skin was accepted whereas skin from a second member of the breed was rejected.

Earlier attempts to demonstrate tolerance to heterologous cells and proteins had failed. In 1950 Burnet himself, encouraged by the earlier observations of Traub with congenital atypical choriomeningitis and of Owen on chimaeric cattle, had tried to experimentally verify his hypothesis of embryonically acquired immunological tolerance. He injected 12-day-old chicken embryos with influenza A virus, bacterial virus C16 and human red cells. Although aware that none of the three antigens satisfied the requirement of persistence, he was disappointed in failing to demonstrate acquired tolerance to any of them.

The first demonstration of immunological tolerance to antigens other than transplantation alloantigens was achieved in 1954 by Hanan and Oyama. They injected purified BSA intravenously into pregnant rabbits. The offspring were unable to form antibodies to BSA, although they formed

antibodies against egg-albumin on challenge. The continuous presence of the antigen BSA was demonstrated in these rabbits. Experiments extending these observations were soon reported by Cinader and Dubert, and Dixon and Maurer, both in 1955.

In this review, I will mainly consider experiments involving unresponsiveness to transplantation antigens induced under conditions designed to mimic those under which self tolerance is assumed to occur. Some important considerations arise, however, when different types of antigens are used. Reference to such systems will be made when appropriate.

2. The Nature of Self Tolerance

The ability of an animal to mount a specific cell mediated and/or humoral immune response against a wide variety of foreign as opposed to self antigens is believed to be due to a regulatory learning mechanism. The inability of F1 animals, in contrast to their parents, to respond against their own antigens that are uniquely derived either maternally or paternally argues conceptually for a learning mechanism in differentiating "self from non-self" as opposed to a genetically encoded one. In addition the frequent occurence of autoimmune reactions provides further evidence that the individual has the genetic ability to react against self components immunologically.

Although there are several experimental examples where the individual's immune system acquires the ability to become tolerant to foreign antigens, the first experimental system to demonstrate that self antigens can be reacted to as though they are foreign was developed by Triplett with the tree frog, Hyla Regilla (Triplett, 1962). The experiments consisted of removing the buccal component of the hypophysis and growing the animal and the gland seperately. When the animals became immunologically mature, the cultured glands were grafted back. In most cases the animals rejected their own hypophesis. Control experiments excluded the possibility that the immunogenicity of the

graft had changed during culture. The same type of experiment, that is the abolishment of tolerance due to the removal of an organ early in ontogeny and its reintroduction later, was performed in FP and SC strains of chickens. It was shown that chickens bursectomized during an early stage of embryonic development were able to recognize bursal antigens as adults upon immunization with bursal cells. Lack of a response on immunization with thymus cells excluded the possibility that a response to minor histocompatibility antigens was responsible for the observed immunity (Grebeneau et al 1976).

Finally, strong evidence for a learning mechanism came from the experiments of Boyse and his collegues in mice. Lethally irradiated C57 mice were reconstituted with (C57XA)F1 bone marrow and spleen cells. The lymphocytes of the chimaeras so obtained were of donor origin. 100% of these animals were able to reject A strain skin grafts after a certain period of time. The rejection was shown to be mediated through the sk alloantigen present on epidermal cells. It was concluded that upon transfer to C57 recipients the hybrid lymphocytes lost contact with the A strain sk antigen and thus recovered their ability to reject A strain skin. (Boyse et al, 1970).

This thesis is concerned with the phenomenon of immunological tolerance, defined as specific immunological unresponsiveness induced by exposure of the embryo, or the

very young immunologically undeveloped individual, to a given antigen, in particular to transplantation alloantigens. By examining mechanisms of phenomena induced during the developmental period of the organism, a period during which non-reactivity to self antigens is acquired, one would hope by extrapolation to explain the mechanisms underlying induction and maintainance of tolerance to self antigens.

The term tolerance was originally used to describe only those unresponsive states induced under an "adaptive" developmental period. Since 1959 it has frequently been used in a broader sense to include all types of induced immunological non-reactivity, irrespective of the state of development of the host's immune system at the time of induction (Chase, 1959). Although there is substantial evidence that the cellular basis of unresponsiveness induced in adults is different from that induced in an embryo or young incompetent individual (Chase and Battisto, 1959; Turk and Humphrey, 1961; Parish and Liew, 1972; Waters et al, 1979) the relevance of different types of unresponsiveness to the mechanism of self tolerance is much debated, (see later under 5). Certain requirements have to be fulfilled in order to obtain a valid experimental model of self tolerance and I shall now discuss these.

3. Requirements On Experimental Systems Used As Models of Self Tolerance

A. The time during ontogenic development that antigen is administered

Immunological tolerance acquired during the developmental stage of an individual is a general phenomenon among vertebrates. Tolerance has been found or experimentaly induced in all species of mammals and birds investigated as well as in amphibians. The purpose of this section is to discuss the length of the developmental period during which tolerance to foreign antigens is easily induced by the administration of a dose of antigen that would evoke a specific immune response in an immunologically mature individual. (The mechanism by which such tolerance is established will be discussed under the relevant section).

Tolerance to transplantation alloantigens has been found to be spontaneously induced as a result of natural embryonic parabiosis. In this case stem cells are exchanged between fetal partners due to vascular anastomosis. Such offspring generally display cellular chimaerism during their whole life and permanently accept skin grafts exchanged between the former partners. Red cell chimaeras are a frequent finding in cattle (Owen, 1945). Although the phenomenon is rare, cellular chimaeras, mutually tolerant of

each other's skin, have been described in twin chickens that arise from double-yolked and doubly fertile eggs (Billingham et al, 1956). Another example of spontaneously induced tolerance most probably occurs when foreign antigens of maternal origin can cross the placenta and gain access to the foetus in sufficient amounts. An example of this type of naturally occuring tolerance involves the allotypic antigens present on immunoglobulin molecules. Mice born of allotypically different parents are tolerant of the maternal 1gG2a allotype which they do not synthesize but have received through the placenta (Warner and Herzenberg, 1970). Tolerance to Lymphocytic Choriomeningitis Virus in mice (Traub, 1939) and to Avian Leucosis Virus in chickens (Rubin et al, 1962) after congenital infection are additional examples.

To define the ontogenic period during which tolerance is easily induced from reports in the literature is difficult as several important parameters are not systematically varied. Information is available concerning the time during ontogeny when: i) induction of unresponsiveness to transplantation antigens can be induced by administering allogeneic cells. The tolerance is assessed by acceptance of skin grafts of donor origin in the adult; and ii) induction of unresponsiveness to purified proteins can be induced upon the administration of a given amount of antigen. The tolerant state is assessed by the absence of humoral antibodies in the serum of the treated animals after

challenge as adults. It is difficult to make a comparison between these two kinds of experiments as both the nature of the antigen and the system of testing tolerance are so different. In the first case the ontogeny of cell-mediated immunity is of importance whereas in the second the ontogeny of the humoral response is relevant. The importance of a system in which tolerance to different types of antigens, at both the cell-mediated and humoral level, is evaluated was recognized and emphasized long ago (Hasek et al, 1961). This comparative type of study is rare probably due to the limitation of test systems available in different species.

In a series of experiments in mice (Billingham et al, 1956), a standard adult tissue preparation from A strain mice was administered to fetal CBA mice at two stages of gestation and at birth. The success in inducing tolerance to A strain skin grafts was 43% when the A strain cells were injected early in gestation and dropped to 23% and 8% for the later injections. Although these experiments lack precision in that the number of cells administered is not known, and are complicated by the fact that the adult A type lymphoid cells injected are able to mount an immune response against the young CBA mice, they are highly indicative of the importance of the time during ontogeny at which the attempt to induce tolerance is made. More elaborate experiments performed by the same group confirmed the initial results (Billingham and Brent, 1959).

In chickens, the species used in the work described in this thesis, the developmental period during which acceptance of allografts is successfully induced ends a few days after hatching. This was shown in the original experiments of Cannon and Longmire as described in the introductory section (Cannon and Longmire, 1952). Another set of observations from this thesis suggests that the period for successfully inducing unresponsiveness to antigens responsible for GVH reactions, another form of cell mediated immunity, ends before hatching (see results). Hasek, in his pioneering studies, attempted to test for tolerance to an antigen at both the cellular and humoral levels. These conceptually original experiments unfortunately suffered from a lack of definition of the antigen employed. He was able to show that adult birds, after successful embryonic parabiosis, are unable to form agglutinins against each others red cells upon immunization (Hasek, 1953). These birds are also able to retain for life skin grafts exchanged between the former partners (Hasek, 1954).

The developmental period during which tolerance to different antigens can be established, and which presumably reflects the level of maturity of the host's immune system, varies among species, strains and individuals. Examples of this variation are the inability to induce transplantation tolerance in rabbits after the 22nd day of gestation (Porter, 1960), the full gestation period being 31 days, and

the difference in susceptibility to tolerance induction shown by neonatal mice of various strains (Holan et al, 1978).

Unresponsiveness at the humoral level to purified protein antigens was studied thoroughly by Smith and Bridges in rabbits (Smith and Bridges, 1956; 1958). In these experiments the same dose of antigen, 100 mg BSA, can induce either tolerance or immunity depending on whether it is given during the first two weeks after birth or later. 100% of the rabbits injected on the first or third day of life were specifically tolerant to BSA when challenged at 4 months of age. The degree of success in inducing tolerance dropped as a function of age with the result that only about 20% of the animals injected at the 17th day of life were unresponsive.

Tolerance at both the delayed type hypersensitivity and humoral level was investigated simultaneously in the experiments of Turk and Humphrey in guinea pigs. Animals injected pre or neonatally with BSA or HGG were unable to form antibodies or display DTH reactions to these antigens upon challenge (Turk and Humphrey, 1961).

As emphasized earlier, it seems that valuable information could be obtained if different types of antigens are compared for their ability to induce tolerance at both the cellular and humoral level at different stages of the ontogenic development of the host's immune system. Such an

approach would provide not only information on whether the time during which tolerance can be induced is different for different antigens but might also suggest reasons for the difficulties encountered in trying to induce tolerance to some bacterial antigens.

In the next three paragraphs I shall briefly describe two phenomena which were observed during the course of experiments on transplantation tolerance and which exemplify some of the complexities that arise in attempts to obtain a valid model of tolerance.

Partial Tolerance was described initially by Medawar and his collegues as an intermediate hyporeactive state between complete tolerance and normal reactivity. In their initial publication they expressed the view that tolerance is not an "all or nothing" phenomenon but is quantitative (Billingham et al, 1953). In terms of allogeneic skin graft survival, partial tolerance was defined as a significantly prolonged retention period of skin grafts which displayed symptoms of chronic rejection. The initial observations were expanded and confirmed later (Billingham et al, 1956). In these experiments they demonstrated that a proportion of animals that carry chronically rejected skin grafts are able to vigorously reject a second graft from the same donor. This is in contrast to fully tolerant animals which were able to retain a second graft for an indefinite period of time. One would anticipate that animals partially tolerant

in the above sense must have antigen reactive cells as opposed to those that are fully tolerant. This type of analysis was made by Brooks. He showed that administration of a limited number of adult (CBAXA)F lymphoid cells to CBA newborn mice led to a partially tolerant state. These animals chronically rejected skin grafts of the donor type. Their spleen cells showed low but significant levels of MLC reactivity (Brooks, 1975). Similar experiments have been described in rats. Rats injected neonatally with adult F1 lymphocytes could be divided into three groups, those in which complete tolerance was established, others that showed partial tolerance and others in which responsiveness was normal. Animals belonging to the second category displayed signs of slow graft rejection and had detectable levels of MLC as well as GVH reactivity against antigens of the donor strain (Heron, 1973). Comparable observations were made by Bernstein et al., (1975). Although the role of specific humoral antibodies in these experiments was not well defined, it was found that sera from animals undergoing graft rejection are able to inhibit the in-vitro MLC reactivity of normal cells (Brooks, 1975). Partial tolerance has also been described at the humoral level. In other words, animals treated with a given antigen display decreased but significant titers of serum antibodies as compared with untreated control animals. Such an example occurs in the experiments of Simonsen in which chicken embryos were injected with adult goose blood. When the

hatched chickens were challenged at five or six weeks of age with goose blood of the original donor, decreased titers of agglutinins against the donor red cells were observed (Simonsen, 1955).

Split Tolerance refers to the situation in which complete tolerance to an antigenic structure of a given complex is induced leaving the responsiveness to another part of the complex unchanged. The phenomenon was first decribed in mice (Billingham and Brent, 1959). Injection of adult (C57xCBA)F cells into newly born A strain mice made them tolerent to CBA but not to C57 antigens as judged by the acceptance of CBA but not C57 skin grafts. The same experiments were further repeated and extended (Brent and Courtenay, 1961).

Although split tolerance is well defined as tolerance to a limited part of a complex antigen the phenomenon is sometimes confused with *immune deviation*. This refers to a state of unresponsiveness at the cell-mediated level that is associated with a state of humoral immunity, and vice versa, to the *same* antigen. There are several examples of immune deviation in systems employing protein antigens (Asherson, 1966; Janicki et al., 1970; Parish and Liew, 1972). In terms of cellular antigens evidence for an inverse relationship between cellular and humoral immunity for the same antigen has been described in the following systems: Radiation chimaeras made by reconstituting A mice with (B6XA) bone

marrow cells are generally able to retain skin grafts of B6 origin, which carries the sk.2 allele. Despite the skin retention alloantibodies against the sk.2 antigen are often found (Scheid et al., 1972). When mice, that have the Thy-1 C3H alloantigen are grafted with skin from their congenic partner strain that has the Thy-1 AKR antigen, they generally retain the grafts but produce anti-Thy-1 AKR antibodies (John et al., 1972). Finally, results from this thesis suggest an inverse relationship between cell-mediated immunity, as manifested by GVH reactions, and humoral antibodies directed against the major histocompatibility antigens of the chicken (see results).

B. Antigen concentration and persistence

Both the induction and duration of tolerance are known to depend on the quantity and persistence of the antigen injected. Specific tolerance to cell surface antigens administered in the form of self-replicating, living cells is long lasting. This type of tolerant animal is often a cellular chimaera, as demonstrated in cattle after natural embryonic parabiosis (Owen, 1945), and in chickens after experimental embryonic parabiosis (Billingham et al., 1956). When antigens are administered in the form of non-replicating cells or as soluble antigens tolerance is short-lived and depends on the initial amount of antigen injected. Tolerance in these systems breaks if antigen is not present but can be prolonged indefinitely if the antigen is given regularly.

The importance of the continuous presence of antigen as a requirement for tolerance induction and maintenance was probably first shown in the experiments of Hasek with interspecies blood chimaeras made by embryonic parabiosis (Hasek, 1957). Hens parabiosed with turkeys contain turkey red cells in their circulation for a period that sometimes extends to 8 weeks post hatching. The above birds were unable to form agglutinins against turkey red cells on challenge at this time but responded when challenged at 6 months of age. Another demonstration of immunological tolerance requiring the persistence of antigen comes from the experiments of Mitchison again performed with chickens. Tolerance to allogeneic erythrocytes was induced by injecting newly hatched chicks with heated or heavily irradiated adult allogeneic blood. Tolerance could be maintained by reinjecting blood at intervals or responsiveness appears by allowing the injections to lapse (Mitchison, 1959, 1962).

The use of soluble proteins for tolerance induction provided systems in which both the initial concentration of antigen required to induce tolerance and the concentration required to maintain it could be determined.

Smith and Bridges were able to show that the duration of tolerance in their experimental system was directly related to the amount of antigen given at birth. A single dose of 100 mg of BSA administered intraperitoneally to

neonatal rabbits produced complete tolerance lasting 135-189 days, 10 mg of BSA produced tolerance lasting 90-135 days, 1 mg of BSA produced tolerance lasting 70-90 days and finally 0.1 mg of protein produced tolerance lasting 47-65 days. They also established the minimum dose required to prolong tolerance. A dose of 10 mg of protein, administered at the 45th day to the group of rabbits that had received 0.1 mg of BSA at birth, was able to prolong the unresponsive state in 100% of the experimental animals for another 4 weeks (Smith, 1961). Similar observations were made with rats using a different type of antigen (Nossal, 1965). A single dose of 100 mg of monomeric flagellin from Salmonella adelaida, given at birth, induced complete tolerance on challenge at 10 weeks of age. Thirty mg of the same antigen induced partial tolerance and 10 mg was insufficient to induce any state of unresponsiveness. In the same study a total of 3.2 mg of flagellin administered biweekly over a period of 16 weeks was able to induce tolerance in most of the treated rats 16 weeks after the last injection. A ten fold lower dose, given under the same regimen, produced tolerance in 2/3 of the animals when challenged 16 weeks later. Finally, none of the rats that received a 100 fold lower dose of antigen appeared tolerant. An even lower amount of antigen was required to induce tolerance in new born rabbits in the experiments of Chou et al. Three micrograms of HSA injected at birth was sufficient to induce tolerance in 30% of the experimental animals when challenged 30 days after injection

and in 15% when challenged at 75 days. This amount of antigen corresponds to a blood concentration of 5×10^{-11}M if all the antigen given is assumed to be present in half a liter of blood (Chou et al., 1966).

When the minimal antigen concentration for inducing tolerance in immunologically immature animals is estimated, it seems that two important factors should be taken into consideration. i) The catabolic rate of the antigen in question. It is known that the catabolic rate varies significantly among different antigens. Polysacharides can persist for several months in the tissues without being catabolized (Ivanyi and Howard, 1971). On the other hand serum proteins have a half life of the order of one to a few days. Relevant to the comparative studies of Smith in rabbits is the fact that Ea has a shorter half life than BSA and BSA has a shorter half life than HGG. Equivalent amounts of antigen administered at birth produce tolerance that lasts longest for HGG and shortest for Ea. ii) The route of antigen administration. The effective antigen concentration depends on the route of its administration. Intravenous injection of antigen facilitates the access of the antigen to the primary lymphoid organs compared to other routes.

From the above stated experiments it seems that the molarity of antigen required to induce tolerance is low. Nevertheless it is natural to assume that there would be certain self components whose concentration is below the

threshold required for tolerance induction. In addition there would be antigenic structures not normally accessible to the lymphoid traffic, such as intracellular components or antigenic structures present on certain "privileged sites" of the body (Barker and Billingham, 1977). The above type of antigen would not be able to induce tolerance. As a consequence clones with specificity for some of these antigens should be present in the adult animal and would be induced under appropriate conditions. An example of this is the formation of autoantibodies against autologous thyroglobulin when a cross reactive antigen is administered (Weigle and Nakamura, 1967) or the molecule is modified in order to make it more immunogenic (Weigle, 1965). Finally, an example of a cryptic antigen is that present on corneal tissue. The tissue is immunogenic when transplanted heterotopically but not when transplanted orthotopically (Billingham and Boswell, 1953).

4. Comparison of the Ability of Different Antigens to Induce Tolerance

A. MHC antigens on viable haemopoetic and lymphoid cells

Tolerance to antigens coded by the major histocompatibility complex of a species is easily established when these antigens are administered in the form of viable haematopoietic or lymphoid stem cells to fetal or newborn animals. Adult immunocompetent lymphocytes are also able to confer tolerance provided that they cannot mount an immune response against the host. If they can do so complications due to GVH reactions arise. Such procedures are well established (Billingham et al., 1956; Billingham, 1961). Tolerance induced by this means is generally haplotype specific in that it covers all cellular antigens coded by the MHC of the species. This aspect is particularly well documented in mice where the MHC is well characterized. H-2, the major histocompatibility complex of mice, is a genetic region defined as being between two loci K and D. These two loci code for cell membrane antigens that are easily recognized serologically. Close to the K end of the complex is the I region. The products of this region are chemically different from the K and D antigens, they are expressed on the surface membrane of some lymphocytes and they have also been identified serologically. The same region that codes for I- associated antigens also controls

the level of the immune response to a variety of antigens.

Antigens coded by the K and I region are responsible for graft rejection. Grafts exchanged across D region differences are also rejected but more slowly. Although antigens coded by the K and D ends can stimulate MLC reactions, I region antigens are responsible for the strongest reactivity. The same holds true for GVH reactions. Although both K and D end products can stimulate GVH reactions the I region products stimulate the strongest response. A possible in-vitro analogue of the allograft rejection is the generation of cytotoxic effector T cells able to lyse cells carrying the appropriate antigens. Such killer cells are easily generated against K and D products but they are less frequent against LPS stimulated blast-cell targets carrying I region associated determinants (reviewed by Klein, 1975).

In the experiments of Beverly et al., (1973) and Brooks (1975), the injection of (CBAXA)F1 cells into newborn CBA mice made them tolerant as judged by three criteria:
i) The indefinite acceptance of donor type skin grafts.
ii) The inability of lymphocytes from tolerant mice to generate cytotoxic cells against the donor type antigens and iii) the absence of MLC reactive cells in the spleens of tolerant animals.

The ability of the antigens, coded by different loci of the H-2 complex, to induce transplantation tolerance was

studied recently by Holan et al., (1978). Tolerance in their strain combinations was induced easily across D and I region differences. K region differences seemed to be resistant to tolerance induction. It seems that there is a disparity between these results and the ones obtained by Brooks (1976) where differences across the I region seemed to be the strongest barrier for tolerance induction. It is reasonable to assume that the reported discrepancies are due to the genetic background and the immunological maturity of the host at the time of antigen administration. In the experiments by Holan et al., for example, different background strain combinations were used when K, D or I region differences were compared. The importance of the host's genetic background for tolerance induction, irrespective of the influence of the different regions of the H-2 complex, was shown in the experiments of Streilein and Klein (1977).

In chickens the first locus, in the genetic region known today to be the MHC of the species, was identified in 1948 as coding for erythrocyte antigens (Briles and McGibbon, 1948). That the B locus was the major histocompatibility system of the species was demonstrated by Schierman and Nordskog (1961). They exchanged grafts among individuals differing at some of 4 erythrocyte antigen systems A, B, D and L. They established the B antigens as responsible for rapid skin rejection. Eventually B-locus antigens were identified as responsible for major GVH-

splenomegaly reactions (Jaffe and McDermid, 1962), major GVH-CAM pock reactions (Schierman and Nordskog, 1963) and mixed lymphocyte reactions (Skopinka and Skamene, 1968; Miggiano et al., 1974). In addition the B locus, or a locus closely linked to B, controls the GVH competence of donor lymphocytes (Longenecker et al., 1972), and the level of the immune response to certain synthetic antigens (Gunther et al., 1974; Kock and Simonsen, 1977). An allele (B^{21}) of the B locus is also associated with resistance to Marek's disease, a herpes-virus-induced lymphoma of chickens (Longenecker et al., 1976; Briles et al., 1977). The genetic region around the B locus is regarded as the MHC of the species, (reviewed by Pazderka et al., 1975). Recently a probable crossing over event inside the B region was reported (Hala et al., 1976). According to the authors the inheritance of B antigens is governed by two loci, the B-F and B-G loci. The B-F locus determines the serological and histocompatibility antigens. The B-G locus codes for antigens that are only serologically defined and appear to have no effect on rejection of skin. The current concept of the structure of the B complex is described by Pink et al., (1977). They proposed a three locus model for the major histocompatibility system of the chicken based on serological and biochemical analysis of the recombinant haplotype reported by Hala et al., and of another recombinant strain derived from the same type of crossing over event. The model proposed suggests that the B-F region

includes two loci, referred to as B and B-L. The models can be described thus:

```
(Hala et al., 1976)
      B-F              B-G
---------------    ---------
    B     B-L          B-G
(Pink et al., 1977)
```

The B-F region contains the B and B-L antigens responsible for skin graft rejection, MLC and GVH reactions. The B antigens are glycoproteins of 40-45,000 M.W. and they are present on both lymphocytes and erythrocytes. The B-L antigens are present only on lymphocytes and their M.W. is around 30,000. The B-G region codes for antigens present only on erythocytes and they are not involved in skin rejection.

In view of the fact that the chicken was the species first used extensively for tolerance induction, it is surprising that there are only a few reports on tolerance to antigens of the B region using genetically defined strains. Completely inbred strains are not commercially available and this is probably partly responsible. Highly inbred lines of chickens do however exist. These are the CB and CC lines of a congenic pair differing at the MHC, described by Hasek et al., (1966) and the WA and WB lines described by Hala et al., (1966). Tolerance to B locus antigens, induced embryonically, was reported by Droege and Mayor (1975) who used the genetically B-region defined strains FP and SC. In this study tolerant birds were able to retain grafts of the donor strain. The ability of lymphocytes from the tolerant

birds to induce GVH reactions was highly reduced, provided that tolerance was induced during embryogenesis. Recently an attempt has been made to evaluate the role of the different antigens of the B region in tolerance induction. These results are reported in an abstract of a Symposium on Laboratory Animals. As such they are limited in nature and thus are not definitive (Hartmanova et al., 1977). Injection of RBC at hatching induced tolerance to red cells in the injected chicks, which were able to reject skin grafts from the donor strain. In contrast the injection of white blood cells elicited prologation of graft survival but did not induce unresponsiveness to red blood cells. In addition, tolerant birds bearing skin grafts had normal GVH reactivity. It thus appears that red cells cannot induce transplantation tolerance although they carry the B-F antigens. This result is indicative of a requirement for a lymphocyte, as opposed to red cells, to induce tolerance to skin grafts, although the opposite result has also been reported, in which red cells injected at hatching are able to induce transplantation tolerance (Kinsky and Mitchison, 1963). In view of this apparent contradiction, Hartmanova's observations require confirmation or Kinsky and Mitchison's interpretation requires a re-evaluation.

B. MHC antigens in other forms

There is considerable evidence that the MHC antigens expressed on viable lymphoid cells are "qualitatively" stronger antigens in eliciting a strong primary cell-

mediated immune response than the same antigens expressed on cells other than viable lymphocytes (Lafferty et al., 1974; Wagner et al., 1975). These findings have led Lafferty to propose that allogeneic interactions constitute a novel class of immune reactivity in which an alive metabolically active lymphocyte or macrophage like cell is required to provide an inductive stimulus to the responder cell in addition to the antigen-receptor interaction (Lafferty et al., 1972). An alternative explanation has been proposed by Rollinghoff and Wagner (1975). They postulated that non-lymphoid cells bearing alloantigens lack determinants able to induce efficient helper cell populations, which in turn are necessary to induce non-memory precursor cells of cell-mediated immune responses. They explained the ability of non lymphoid cells to induce a secondary response by postulating that memory cells are induced in the absence of help. An alternative explanation for the ability of non-lymphoid cells to induce secondary as opposed to primary responses is that the induction of memory and virgin cells both require help, but that the higher numbers of helper cells present in primed cell populations allows non-lymphoid cells to induce a response (Pilarski, 1979). The functional role of MHC antigens on lymphocytes is beyond the scope of this review, although it seems reasonable to keep in mind the possibility that their observed behavior in eliciting a cell-mediated immune response might parallel their ability to induce cell-mediated tolerance. When non-reproducing cells, or lymphoid

membrane fractions, are used to induce tolerance quantitative considerations come into the picture. There are only a few reports showing that MHC antigens administered either on cells other than live lymphocytes, or as soluble antigens, can induce tolerance.

Initial attempts to induce transplantation tolerance in new born mice or in newly hatched chicks by administring crude cell-free extracts failed (Billingham et al., 1958). The same negative results were obtained by Haskova (1959) both in chickens and in ducklings. Administration of allogeneic red cells, free of lymphocytes to chicken embryos was unable to induce tolerance to skin transplants although it led to unresponsiveness at the humoral level as judged by the absence of red cell agglutinins in the sera of treated birds (Hasek, 1957). More elaborate experiments along this line have been performed recently in mice. In these studies H-2 antigen from the spleen cells of A/J mice was solubilized and partially purified. It was shown that the preparation contained the major H-2 antigenic determinants detected _in situ_. Administration of repeated doses of soluble H-2 antigen to newborn B10.D2(H-2d) mice prevented the production of cytotoxic, haemagglutinating and enhancing antibodies upon challenge with lymphoid tissues of donor origin. Nevertheless these mice had normal or enhanced levels of cell-mediated immune functions as judged by several criteria; they rejected skin grafts, had normal levels of MLC and GVH reactivity and their lymphocytes could

be induced to produce cytotoxic T cells (Law et al., 1971; 1972; 1973, 1974). A different result was obtained in a series of experiments in rats. Partial transplantation tolerance was induced to the strong H-1 and the less strong non H-1 histocompatibility antigens that are present in the sera of normal animals. By injecting excessive amounts of allogeneic sera to newborn rats they succeeded in inducing specific tolerance to skin transplants when the weak non H-1 histocompatibility differences were involved. The procedure had only limited success with strong H-1 differences. Interestingly enough the experimental animals that rejected the transplanted donor type skin were unable to form humoral anti donor strain antibodies (Hasek et al., 1976; Chutna et al., 1977).

C. Other cellular antigens

It is most helpful, when studying the mechanisms underlying tolerance induction to differentiation antigens present on cell surfaces, if polymorphic forms of the particular antigen occur within the species. The alloantigen sk present on skin cells of mice exists in two allelic forms, sk-1 and sk-2. In the experiments of Lance et al., (1971) lethally irradiated C57BL/6 mice, carrying the alloantigen sk-1, were reconstituted with bone marrow (C57XA)F1 cells. A mice carry the sk-2 alloantigen. Neither bone marrow cells nor their progenitors bear the sk alloantigen. The chimaeras described above were unresponsive to the sk-2 alloantigen for a period of 5 weeks. Reactivity

against the antigen sk-2 as measured by skin rejection was progressively acquired after the 6th week. This period is presumably the time required to generate sufficient new precursors cells with specificity for the sk-2 alloantigen in the new, antigen free, environment. A similar type of result was obtained with the liver specific differentiation alloantigen F which exists in two allelic forms I and II (Fravi and Lindenmann, 1968). Mice of type FI were repopulated with bone marrow cells of type F I/II. Tolerance in these chimaeras was maintained by weekly injections of low concentrations of antigen, 10 mg of F II protein, (unpublished results of Winchester, quoted by Mitchison, 1978). Mice are generally tolerant to the exposed part of the self F antigen, but they can form antibodies directed mainly against the invariant intracellular part of the molecule when immunized with liver homogenates from strains carrying the alternative allele. Mice can be rendered tolerant to the alloantigen F by neonatal administration of allogeneic spleen cells (Iverson and Lindenmann, 1972). Presumably the F antigen is present on some splenic cell. Another type of cell-surface antigen which has been used in studies of tolerance induction is the H-Y antigen. The expression of this antigen occurs only on male cells although is not clear whether it is structurally coded for, or its expression is regulated by, the Y chromosome (Reviewed by Wachtel, 1977). Within a highly inbred strain female mice as a rule reject skin grafts of male origin. In

a series of experiments in mice Billingham and Silvers (1958, 1960) succeeded in inducing tolerance to the H-Y antigen present on skin by injecting adult C57 male spleen cells, or bone marrow male cells from three different strains, to newborn C57 female mice. Interestingly enough the post birth period during which mice were susceptible to tolerance induction with this fairly weak transplantation antigen extends to a period of 17 days after birth.

Erythrocyte antigens of allogeneic or xenogeneic origin have been a major experimental tool in immunology. Erythrocytes carry a wide spectrum of cell surface antigens such as blood group, histocompatibility and probably several unidentified species specific and differentiation antigens. The concentration of these different types of antigen on the cell surface varies among different species. For example, histocompatibility antigens are poorly expressed on mouse red cells (Klein, 1975), while the same class of antigens are highly expressed on nucleated chicken erythrocytes (Pasderka et al., 1975). Another source of variation is the existence of natural immunity in a given species against antigenic determinants present on the red cells of another species. For example, natural agglutinins are sometimes present in the sera of normal unimmunised animals and PFC specific for xenogeneic erythrocytes can be found in the spleens of uninjected animals.

The preexistence of strong natural immunity, the

heterogeneity of surface antigens, and the fact that these cells are non-replicating should be taken into consideration when comparisons are made between the ability of different types of erythrocyte to induce tolerance. These parameters were taken into consideration by Mitchison in his studies on the induction of tolerance in chickens (Mitchison, 1959; 1962). Tolerance to erythrocyte alloantigens, as measured by the inhibition of agglutinin production, was induced by repeated administration of heavily irradiated (10,000 r) adult allogeneic blood into embryos or newly hatched chicks. Complete tolerance induced by this means could be maintained indefinitely provided red cells were regularly administered. Injection of irradiated adult turkey blood into young embryos or newly hatched chicks resulted in a group of animals displaying variable degrees of tolerance including complete unresponsiveness as judged by the lack of Cr^{51} labelled red cell elimination from the circulation of the treated animals. It was observed during these experiments that xenogeneic cells could induce tolerance if given at a certain early period of incubation. The author correlated the resistance in tolerance induction with the presence of naturally occuring antibodies against the turkey cells, passively transferred from the hen to the egg. Indeed after the 14th day of incubation turkey red cells are rapidly eliminated from the circulation of the embryonic chick as opposed to allogeneic chicken erythrocytes. The time at which turkey red cells begin to be rapidly eliminated

coincides with the time that antibodies injected into the yolk pass into the circulation (Schechtmay and Knight, 1955). In addition, naturally occurring haemaglutinins against turkey red cells are first found in the embryonic blood at the 16th day of incubation (Ryle, 1957). According to Mitchison the existence of natural antibodies transferred from the mother to the embryo is responsible for neutralizing the antigen and preventing induction of complete tolerance. An alternative explanation but along the same lines is that helper factors, passively transferred from immune mothers to fetuses, allow an early induction of anti-xenogeneic red cell responses. For example, Rowley and Fitch (1965) were able to detect plaque forming cells in the spleens of 3-day-old normal rats against sheep red cells. The same findings could also be interpreted as an endogenous response to self red cell break-down products which cross react with sheep red cell antigens in analogy to the findings in mice (Cunningham, 1974). This type of response does not appear in the mouse, however, until the second week after birth and reaches adult levels by the end of the third week (Cunningham, 1976). Another interesting feature of the experiments by Kinsky and Mitchison was the successfull induction of transplantation tolerance by administration of heavily irradiated adult whole blood. Exposure of cells to 10,000 r did not impair the ability of chicken red cells to induce tolerance to skin grafts in chickens. The same dose largely abolished the capacity of separated lymphocytes to

do so (Kinsky and Mitchison, 1963).

D. Soluble protein antigens, viral antigens, and antigens present on common pathogenic bacteria.

Tolerance to different purified soluble antigens or pathogens induced during embryogenesis or immediately after birth is an extremely extensive subject and is beyond the scope of this review. Instead, an attempt will be made to give a general description of the observations and to point out some cases in which induction of tolerance to antigens present on common pathogenic bacteria appears difficult to achieve. Where possible a comparison will be made among species with reference to a given antigen, between different routes by which the antigen is administered to the animals, or between different times during ontogenesis that the antigen is administered. In some cases the importance of naturally occuring immunity to an antigen will be discussed.

Tolerance induced by soluble serum proteins in embryos or very young animals has been mainly demonstrated as a specific inhibition of antibody formation against the antigen employed. In these studies heterologous serum albumins and serum immunoglobulins have often been used to induce tolerance in several species. It is probably worthwhile mentioning here that tolerance to serum proteins in most of the initial studies was achieved by injection of the antigen into pregnant animals or directly into embryos. The successful transfer of the antigen from the mother to

the fetus was ascertained in these studies Humphrey and Turk, 1961; Tempelis and Wolfe, 1958; (Humphrey 1964).

Tolerance to protein antigens was first demonstrated in rabbits as described earlier (Hanan and Oyama, 1954). Subsequent studies in this species established the amount of antigen required and the period after birth in which tolerance is easily induced (Smith and Bridges, 1958). 10 mg of BSA, HGG, ovalbumin or human macroglobulin injected at birth rendered most of the animals tolerant upon challenge with two intravenous injections of 10 mg of protein between 90-120 days of age. Tolerance was demonstrated by the absence of serum antibodies in the treated animals.

In mice, tolerance to the serum protein BSA was first demonstrated by Terres and Hughes (1959), as a failure of the newly born treated animals to develop anaphylactic sensitization to BSA or to form antibodies against this antigen. Both the degree and the duration of the tolerant state depended on the amount of antigen given during the early post birth period. In another study specific tolerance to BSA, by neonatal administration of different doses of antigen, was characterised (Sercarz and Coons, 1963) as absence of specific serum antibodies and absence of cells in the tolerant animals having specificity for BSA. Antibody containing cells were stained with antigen, washed and then overlaid with fluorescien-labelled anti-BSA serum (Sercarz and Coons, 1963). Finally the same result was obtained by

Dietrich and Weigle (1963). In their extensive studies which employed 13 different serum proteins, albumins and immunoglobulins from 6 different species, tolerance was produced by a single injection of protein in newborn C57 mice. Specific tolerance was assessed by the rate of elimination of labelled antigen on challenge at different times after birth.

Specific tolerance at the humoral level was induced in chickens upon administration of the same dose of BSA at the 15th day of embryogenesis or at hatching. Eighty percent of the treated birds were tolerant to a subsequent i.v. challenge of the antigen and the remaining 20% displayed partial tolerance (Tempelis et al., 1958).

A thorough analysis of tolerance to HSA, induced in newborn goats, was performed by Carter and Cinader (1960). In their studies they found that most of the neonatally treated animals developed an antibody against their antigenic preparation when challenged as adults. They were able to characterize this antibody as generated against a specific impurity, haptoglobin, present in very small amounts in their HSA preparation. When challenged with high concentrations of this antigen, the goats made antibodies against haptoglobin although it was established that specific anti HSA antibody was not induced.

Long lasting tolerance to HGG and BSA was induced _in-utero_ or at birth in guinea pigs. The availability of assays

in this species to detect DTH reactions made these studies even more interesting. It is probably the first protein system in which specific tolerance to an antigen was detected at both the cellular and humoral level. The treated animals specifically failed to eliminate the antigen or produce antibody or have DTH reactivity on challenge as adults with these antigens in a water in oil emulsion. Interestingly enough a group of tolerant animals developed antibodies against HGG when challenged with this antigen in CFA without a detectable phase of DTH reactivity, (Humphrey and Turk, 1961; Turk and Humphrey, 1961). It has recently been reported that the administration of HGG, BSA or a haptenated synthetic polypeptide to pregnant Balb/c mice results in unresponsive offspring only with the antigen HGG. These results appear to suggest that tolerance cannot in general be induced in mice, as opposed to other species as already described, by administering soluble antigens _in-utero_, (Waters et. al., 1979).

It is difficult to induce tolerance to a certain class of highly immunogenic antigens present on common pathogenic microorganisms, by pre- or neonatal exposure of the animal to these antigens. An early and effective immune response against these pathogens is probably of survival value for the species. Clones with specificity for some of these antigens may arise very early in development and occur with a relatively high frequency. In addition, embryos may achieve immunological competence to these antigens

particularly early due to some maternal influence. The case already described and explored by Mitchison (1962) using turkey blood in chickens could be a model for this. Both the early administration of the antigen and its concentration may be particularly crucial in these cases for inducing tolerance. In addition, naturally occuring bacterial flora, which generally colonize the species by the first week after birth, produce a constant source of antigenic stimulus and normal animals may regularly display a continuous immune response without deliberate administration of antigen. In this context, passively transferred immune molecules from the mother to the fetus could prevent some antigens from inducing tolerance. In the next few paragraphs, I will discuss a series of experimental systems where both positive and negative results were obtained with antigens present on pathogens, and to examine cases where the above considerations were taken into account.

Burnet, in his attempt to verify his theory of embryonically acquired tolerance, failed to do so with influenza virus by allantoic inoculation of chicken embryos and with the bacterial virus C16 by yolk sac inoculation. Six week-old chickens, treated in that manner as embryos, were able to respond to these antigens when challenged (Burnet et al., 1950). Schwartz et al., (1964) reported that inoculation of pregnant guinea pigs or newborns with influenza and vaccinia virus resulted in a group of animals displaying partial unresponsiveness to the viruses. In the

same study, mice infected at birth with influenza virus were unable to form haemaglutinating or neutralizing antibodies. As reported in the historical introduction, tolerance to another virus, LCM, was established in a colony of mice over a period of 4 years, with congenitally transmitted virus (Traub, 1939). The same experiments were repeated later (Seamer, 1965). In chickens tolerance to avian leucosis virus, RIF, acquired by congenital transmission, has been described by Rubin et al., (1962) (Qualtiere and Meyers, 1979). Chickens that have been exposed to virus by this means have viraemia as adults in the absence of humoral antibody. Control adult birds injected with RIF show a limited degree of viraemia and have humoral antibodies against the virus. Tolerance was specific in the sense that RIF virus tolerant birds were able to form antibodies against influenza virus when injected with this agent. Tolerance to certain pesistent but not strongly pathogenic viruses is reviewed by Volker and Laseu (1965).

Attempts to induce tolerance to different types of polysacharides have also produced mixed results. Siskind et al., (1963) were unable to induce tolerance to type II pneumococcal capsular polysacharide in mice. Medium doses of antigen induced humoral antibodies in both newborn and young adult animals. The same high dose of antigen resulted in mice of both ages being unresponsive at the humoral level. The same negative results were obtained in rabbits. Killed pseudomonas NCMB 406 not only failed to induce tolerance

upon injection to newborn rabbits but actually primed these animals. The authors observed a typical secondary response in their experimental animals when they challenged them as adults (Gowland et al., 1965). Finally, the administration of dextran B135 and levan in newborn mice failed to induce tolerance (Howard and Hale, 1976). In contrast to this result, Friedman and Gabby (1960a, 1960b) and Friedman (1966) reported a series of experiments in chickens and mice in which they were able to induce tolerance to a carbohydrate antigen. Administration of killed Shigella paradysenteriae into chicken embryos led to specific long lasting but not complete tolerance. Intravenous injection of the antigen between 15 days of embryogenesis and hatching induced tolerance better than intramuscular or yolk sac inoculation. Administration of soluble shigella antigen, 50% of which was of carbohydrate nature, into newborn mice rendered them tolerant at the humoral level as measured by the specific absence of PFC in the spleens of tolerant mice.

Smith and Bridges (1958), in the same series of experiments where tolerance to serum proteins was studied, examined whether tolerance to a wide spectrum of antigens present on common pathogens could be induced. Administration of diphtheria toxoid, typhoid paratyphoid, Group A streptococcus, tuberculin, BCG and bacterial endotoxin, all failed to induce tolerance. In contrast different results were obtained by Buxton (1954). Intravenous administration of Salmonella pullorum to chicken embryos at the 15th day of

embryogenesis led to inhibition of antibody formation upon challenge at 80-100 days after hatching. Administration of the same antigen at day 20 of embryogenesis failed to induce tolerance. It should be pointed out that high titers of anti-salmonella agglutinating antibodies, passively transferred from the hen, are found in the embryonic circulation after the 16th day of incubation, (Buxton, 1952). A similar result was obtained in cattle, which were unable to form antibodies for a long period of time to killed Trichomonas fetus, when this antigen had been administered in relatively high amounts to newborn calves (Kerr and Robertson, 1954).

A very interesting study was performed by Weiss and Main (1962) in guinea pigs. They attempted to determine precisely the time at which a pathogen-derived antigen must be administered during embryogenesis in order to induce tolerance. Unfortunately the results are too limited to draw definite conclusions. Guinea pig fetuses were directly injected at different embryonic stages with diptheria toxoid and 4 weeks after birth were challenged with the pathogen. No tolerance was induced if the antigen was administered up to a period of 15 days before birth. One third of the animals injected between 19 and 27 days before birth were tolerant. The guinea pig has a gestation period of 60 days. Tolerance was assessed by the absense of flocculating antitoxin antibody after skin testing and immunization. Finally Nossal et al., (1965) were able to induce tolerance

to antigens prepared from the flagella of Salmonella adeleida in rats. Monomeric flagellin, the main protein component of flagella, was able to induce complete tolerance upon injection of newborn rats with a single dose of 100 mg of antigen. Tolerance was assessed as absence of antibodies in the treated animals after challenge. Polymerized flaggelin, resembling structurally the intact flagella, as well as intact flagella, induced different degrees of tolerance from partial to complete depending on the antigen dose used.

5. Cellular Mechanisms Proposed to Explain Tolerance to Self Antigens

I shall make an attempt to describe the more central views on the cellular basis of self-non-self discrimination and subsequently to provide a description of the experimental evidence which supports the different hypotheses presented. Emphasis will be put on experimental systems which employ transplantation antigens for tolerance induction in the different species during the pre or neonatal period of their life.

A. The Clonal Deletion Hypothesis

The hypothesis of elimination of clones with specificity for self antigens was first introduced by Burnet. The basic concept in the formulation of this theory is that the physiologically immature lymphocyte is destroyed when it interacts with antigen. By "physiologically immature" Burnet means a cell bearing few receptor molecules as opposed to a mature lymphocyte that bears a higher number and is capable of being induced on contact with antigen to produce antibody or become a memory cell (Burnet, 1959, 1961).

Lederberg proposed that any mechanism explaining how antibodies are formed should also explain how anti-self responses are avoided. For that, he postulated a time model for tolerance and immune response induction. According to

this theory, lymphocytes, during the process of differentiation, pass through an initial stage of immaturity during which contact with antigen leads to irreversible destruction. Lymphocytes maturing to this stage and encountering self-antigens are destroyed. Lymphocytes that differentiate further are able to be induced to produce antibodies. In this theory the distinction between the ability of an antigen to induce tolerance or immunity depends only on the time that the antigen is introduced to the potential antibody forming cell (Lederberg, 1959).

A particular term, "clonal abortion", was introduced by Nossal and Pike (1975) to describe the destruction of the immature lymphocyte on contact with antigen in contrast to the regularly used term "clonal deletion", which refers to the inactivation of a lymphocyte, irrespective of its maturity, on contact with antigen.

A variation of Lederberg's time model for tolerance induction has been proposed recently. According to this theory IgM surface immunoglobulin receptors appear in the early phases of lymphocyte maturation. Interaction of antigen with lymphocytes bearing IgM receptors leads to their destruction. Further maturation of the lymphocyte and acquisition of surface receptors of the IgD class allows the cell to be induced on antigen interacting with the IgD receptors (Vitetta and Uhr, 1975).

Different requirements for paralysis and induction at

the level of the mature immunocompetent cell were postulated by Bretscher and Cohn in a theoretical analysis of the problem. Their theory was proposed to account for self-nonself discrimination and immune induction in view of the already known facts of T-B cell cooperation and the hapten-carrier effect. The theory postulates that the same cell is paralyzable and inducible. The discrimination is made by the ratio of two types of signals that the cell receives. Paralysis is mediated by a signal delivered to the cell on the interaction of the cell surface immunoglobulin receptors with the antigen (Signal 1). Induction follows on the delivery of both signals 1 and 2. Signal 2 is mediated via an associative antibody carried by a second cell type. Tolerance to self antigens is established by paralysis of the relevant clones as soon as they arise on contact with antigen. The property that discriminates a self from a foreign antigen is its continuous presence which leads to paralysis of both B and T cell clones and diminishes the possibility of their accumulation in sufficient numbers to allow cooperation and induction (Bretscher and Cohn, 1970; Bretscher 1972).

In the context of the one-nonspecific signal theory for B cell induction, Moller has proposed a model to account for tolerance to self antigens. According to this theory, T cells are inactivated by an unspecified mechanism. Self antigens are thymus dependent and as such they are not able to induce B cells, which are only activated when they

receive a mitogenic, non specific signal from T cells. Thymus independent antigens deliver their non-specific signal in the absence of T cells. The discrimination between self and foreign antigens depends on the ability of T cells to be activated by the thymus dependent antigens. In the absence of T cells B cells are not induced and tolerance is assured. In situations where helper activity could be generated through cross reactive antigens, or new helper determinants tolerance breaks and autoantibodies are formed (Moller, 1976).

B. Active Suppression Hypothesis

The basic argument behind an active regulatory mechanism controlling non reactivity to self antigens is based on the existence of clones with specificity for certain autoantigens in normal animals, or on the demonstration of an ongoing immune reactivity against them. Although these situations exist the animals appear clinically normal and autoimmune reactions occur only after deliberate manipulation of the system. Perhaps the most serious challenge to the concept of early deletion of the self reactive clones came from the findings with allophenic mice. These remarkable animals, whose chimaeric state is established at the eight-cell stage (Tarkozoski, 1961), were shown in some cases to contain a class of effector lymphocytes able to kill in-vitro fibroblasts of either parental strain although they clinically appeared to be healthy individuals (Wegmann et al., 1971). Actively

synthesized inhibitory molecules and/or cells are required in these cases for controlling the deleterious effects of strong cell-mediated autoimmune reactions. I will try to classify the types of inhibitory mechanisms that have beem postulated to explain the way that tolerance to self antigens is maintained. Most of the views to be presented came from studies where tolerance to transplantation antigens was established by administering living allogeneic lymphoid cells to immunologically immature animals. The long lasting unresponsiveness in these individuals and the continuous presence of the antigen, without an obviously effective immune response against it, makes this system a central model for an analysis of the cellular mechanisms involved in acquisition and maintainance of tolerance to self antigens.

i) Active control by humoral immunity

Voisin first proposed that in mice long lasting tolerance against living cells is mediated by an active immunological response producing low levels of anti H-2 "facilitating" or "synergistic" antibodies, acting by protecting the target cells from more drastic forms of immunity. He considered this class of immune response as existing between delayed hypersensitivity and humoral immunity which produces complement fixing antibodies (Voisin, 1968). In other words, he accepted that tolerance is mediated by immunological enhancement in the same way that was originally defined by Kaliss (1958) as a successful

establishment and growth of a tumor graft in the presence of specific antibodies directed against the graft's antigens.

An alternative way in which antibodies may act to inhibit an active immune response in vivo was proposed by the Hellstroms (1971). They suggested that the antibody produced combines with shed antigen and that the antigen-antibody complexes so formed inactivate the immune effector lymphocytes by blocking their receptors, in a way analogous to that observed in an in-vitro system in which exposure of lymphocytes to an antigen-antibody complex of a given composition was able to paralyze the immune activity of the relevant clones (Diener and Feldman, 1970).

A very interesting interpretation of the role of antibodies in the maintenance of neonatally established tolerance to transplantation antigens was offered by Ramseier. He suggested that the adult hybrid (AxB) spleen cells, upon injection to newborn (A) mice, are able to recognize and make antibodies against anti-B receptors, present on some parental but not F1 cells. This "anti-receptor" or "anti-idiotypic" antibody is synthesized continuously and prevents the parental cells from destroying the donor type cells. Although this explanation was suggested to account for how chimaerism and tolerance is maintained in neonatally induced unresponsiveness after injection of allogeneic hybrid cells into parental type new born mice, the role of such antibodies as mediators of self

tolerance was excluded on theoretical grounds (Ramseier and Lindenmann, 1975). In spite of this, these results have been widely interpreted as favouring an active mechanism mediating unresponsiveness to autoantigens via antibodies produced against idiotypes present on receptors of cells with specificity for self-antigens.

ii). Active control by suppressor cells

The mechanisms by which suppressor cells may act as inhibitors of anti-self reactivity is usually undefined. Their existence is postulated to be necessary if anti-self reactivity is a regular event. The existence of antibody forming cells (Steele and Cunningham, 1978) and T cell mediated immunity (Ramshaw and Eidinger, 1977) against isologous antigens present on break-down products of self erythrocytes led to the suggestion that an active suppressor control mechanism is necessary (Cunningham, 1976).

The existence of a suppressor cell controlling autoimmune reactions against self antigens present at very low concentration, such as thyroglobin, was postulated by Allison and Denman (1976). They suggested that self antigens existing it low concentrations could inactivate T helper cells but not B cells. In the absence of helper cells, B cells are not induced. As helper cells could be easily generated with a cross reactive antigen autoantibodies in these situations would be easily induced. To avoid autoimmune reactivity a suppressor cell would be required to

act as a further controlling element.

6. Experimental Evidence for Different Models of Self Tolerance

A. Clonal Deletion

The experimental evidence which supports the clonal deletion hypothesis of anti-self reactive clones can be classified under the following categories:

i) Failure to demonstrate activity of lymphocytes with specificity for self antigens or for antigens that have been introduced under conditions that mimic those under which self tolerance is believed to be acquired. This failure of reactivity is shown either *in-vivo* or *in-vitro* as unresponsiveness to a challenge with the same antigen which evokes a strong immune response in untreated animals. In some systems a functional absence of cell mediated and humoral immunity is demonstrated and in others only one of the above classes is analyzed.

ii) The experimentally produced tolerant state can be broken by the adoptive transfer of non tolerant syngenic lymphocytes.

iii) The non-reactive state is not infectious. This is demonstrated as inability of cells from tolerant animals to inhibit the response of normal syngeneic lymphocytes, both *in-vivo* and *in-vitro*, or inability of humoral factors to inhibit the response of normal cells, again either *in-vivo* or *in-vitro*, against the tolerated antigen.

iv) Slow recovery of normal responsivenes after the

clearance of the antigen. If normal numbers of precursor lymphocytes with specificity for antigen exist in an inhibited state one would expect that the animal would acquire normal levels of reactivity once the driving force of the inhibition, the antigen, is removed. Although this argument is regularly used in favor of clonal deletion a prolonged unresponsive state is not incompatible with an inhibitory mechanism.

v) Support for the clonal deletion hypothesis comes from in-vitro experiments in which paralysis of both B and T cells has been demonstrated when lymphocytes from embryonic or very young animals are allowed to react with a particular antigen.

Evidence for deletion of clones with specificity for autoantigens existing at high concentrations come from the following types of experiment. In humans, antigen binding cells with specificity for autologous serum albumin are absent (Bankhurst et al., 1973). Rabbits immunized with the alpha chain of human haemoglobin are unable to form antibodies against structures present on their own haemoglobin (Reichlin, 1972). Mice immunized with isologous immunoglobulin are again unable to form antibodies against idiotypic determinants that they possess (Seppalla and Eichman, 1979).

Evidence for clonal deletion also comes from the absence of antigen binding cells in rats and mice made

neonatally tolerant to the antigen. Lymphocytes from normal mice and mice made neonatally tolerant to BSA were analyzed for their ability to bind heavily iodinated BSA. Normal mice contained a few cells (6-12/10,000 cells) able to bind BSA. In contrast no such cells could be found in neonatally tolerant mice when tested at 38 days of age (Naor and Sulitzneau 1967, 1969). Recently, in a series of studies in rats and mice, the ability of the hapten fluorescein, coupled either to isologous or heterologous carriers, to induce tolerance in neonates and adults was compared (Venkataraman and Scott, 1977; Scott et al., 1979). No antigen binding cells could be detected in both adult and neonatally tolerant animals 7 days post antigen administration. Upon reinjection of the antigen, however, antigen binding cells were found in animals that had been made unresponsive as adults at values equal to that of the controls. In contrast no such cells could be found in the neonatally treated animals. Furthermore, administration of the B cell polyclonal activator LPS to lymphocytes from the former animals was able to yield a good PFC response, in agreement with results obtained by Moller et al., (1976). In contrast, no such reversal of the unresponsive state was achieved in the neonatally treated animals. Deletion of clones in animals made neonatally unresponsive is not always found. Inactivation but not deletion was observed in another system, where tolerance to the flagellin protein from Salmonella adeleida was studied in rats. Neonatally treated

animals contained the same number of antigen binding cells as control animals but upon reimmunization the number of antigen binding cells remained constant in the unresponsive animals in contrast to the dramatic increase in the number of antigen-binding cells in control animals (Ada, 1970; Ada and Cooper, 1971).

As mentioned earlier, those cases where the unresponsive state extends to both humoral and cell-mediated immunity, and where cells or humoral factors cannot be detected, are often interpreted as evidence for clonal deletion. It is assumed that the lack of reactivity in these situations must be due to a simple lack of immunocompetent cells. In some experimental systems of this nature the investigators did not attempt to determine whether inhibitory cells or serum factors were present as these had not yet been discovered.

Chicken chimaeras produced by embryonic parabiosis are able to tolerate skin grafts exchanged between the former partners. The same birds show complete inability to form agglutinins upon challenge as adults with each others blood. Unfortunately, it is not clear in these studies if the responses measured in the various assays were against the same or different cellular antigens, since the birds used were not genetically charactarized (Hasek, 1953; 1954). In guinea pigs long lasting tolerance to BSA and HGG is induced when administered before or at birth at both the humoral and

the DTH level (Humphrey and Turk, 1961; Turk and Humphrey, 1961). In contrast, it has been shown that in this species unresponsiveness to picryl cloride, induced in adult animals, for DTH reactions was accompanied by a strong humoral response against the hapten (Chase and Battisto, 1959). Parallel experiments were performed in newborn and adult rats. Administration of fragment A of flagellin from salmonella adeleida to newborn rats produced complete tolerance for both humoral and cell mediated immunity. In contrast, administration of low and high doses to adult rats made them unresponsive at the humoral level with concomitant DTH reactivity (Parish, 1971; Parish and Liew, 1972).

There are numerous examples of tolerance to transplantation antigens being successfully induced in animals early in ontogeny. These results require careful classification since the absence of cell-mediated immunity in these systems has often been regarded as strong evidence for clonal deletion at least at the T cell level. It should be remembered, however, that in some of these systems humoral immunity has been demonstrated. This observation argues not only against a deletion hypothesis, but against T cell tolerance in general, as the induction of antibodies to transplantation antigens is known to require helper T cells (Lake and Mitchison, 1976). In the remainder of this section I will present the experimental systems which support the clonal deletion hypothesis, not only at the T cell level, but at the level of humoral reactivity.

Tetraparental animals are probably one of the most valuable experimental systems for analyzing basic biological problems in development and differentiation. These individuals are formed by fusing the blastomeres from two genetically different embryos in vitro. These double size blastomeres are transferred to the uterus of pseudo-pregnant mothers, where reduction of the double size to normal embryos occurs during implantation. Such animals are healthy individuals in which two distinct cell populations coexist in most tissues for an entire life time (Tarkowski, 1961; Mintz, 1961). The chimaeric composition of the different tissues can vary dramatically. If the two stem cell populations differ in their rate of proliferation during embryogenesis and/or adult life, it is natural to expect predominance of one cell type over the other. An extreme example of selective advantage of one erythropoetic tissue over the other occurs with the w/w <--> +/+ mouse chimaeras where the +/+ red cells predominate over w/w red cells as w/w haemopoietic precursors are defective (Mints, 1970). A change in the relative proportions of red cells in sheep chimaeras was observed during adult life (Tucker, 1974). Conflicting results have been reported in tetra-parental C3H <--> C57 mice in which C57 erythocytes became dominant over those of C3H in one system (Mintz, 1969) although no change with time was found in the majority of the same type of chimaeras (Wegmann and Gilman, 1970). Tetraparental mice produced between two genetic strains differing at the MHC

were produced and analyzed for tolerance to parental transplantation antigens. All C3H <--> C57 chimaeras tested had both red cell types and were tolerant in the sense that they specifically accepted skin grafts from both parental types over their entire life (2 1/2 - 3 years). Chimaeric animals whose skin colour showed that the pigmented cells were only derived from one parent were able to reject skin grafts from the other type. For example C3H <--> C57 chimaeras which had only the black C57 colour were able to reject C3H skin grafts although such animals were shown to contain C3H bone marrow cells, a situation which strikingly resembles that studied by Boyse and his collegues using the sk alloantigen system and which was described earlier (Mintz and Silvers 1967; Mintz, 1969). C3H <--> C57 allophenic mice whose chimaerism included skin colour and Ig allotypes had lymphocytes which were specifically unable to cause an MLC reaction against both parental cell types. Neither cells nor serum factors were able to inhibit the reactivity of normal cells from one parental type against the histocompatibility antigens of the other type (Meo et al., 1973). Lymphocytes from a series of genetically different allophenic mice were unable to cause GVH reactions against animals having the MHC antigens present in the chimaeras. Sera from the same animals were unable to inhibit the GVH reactivity of normal lymphocytes (Barnes and Graham, 1976). Finally, similar results demonstrating specific absence of MLC reactivity and blocking factors, were obtained in an analysis of the immune

status of a human tetragametic individual (Ceppelini, 1971).

Transplantation tolerance, induced in newborn mice and rats, has been extensively analyzed. In this section those observations which support a clonal deletion hypothesis, in that abrogation of the tolerant state occurs on the adoptive transfer of syngeneic lymphoid cells, will be described.

Beverly et al., (1973) studied tolerance to MHC antigens in CBA mice which were rendered tolerant by neonatal administration of varying doses of (CBAxA)F1 spleen cells. Mice that received 50×10^6 spleen cells at birth had no MLC reactive cells in their spleens and did not generate killer cells against A type antigens. Sera from these animals were also unable to inhibit MLC reactivity of normal CBA lymphocytes against A stimulator cells. In contrast, mice that received 5×10^6 cells at birth generated cytotoxic cells against A type antigens and their sera did not contain any inhibitory molecules. Finally, injection of $12\text{-}15 \times 10^6$ spleen cells at birth resulted in animals that had no killer cells but contained in their sera factors able to inhibit the MLC reactivity of normal CBA lymphocytes against A stimulators. Similar results were obtained by Brooks (1975) using the same strains of mice. A search for suppressor cells in this study was unsuccessful.

Abolition of tolerance, by transfer of normal or sensitized syngeneic lymphocytes to adult mice and rats that had been rendered tolerant neonatally, was originally

demonstrated by the rejection of skin grafts carried by the experimental animals. Tolerance could also be abrogated by parabiosis of the tolerant animals to normal syngeneic animals (Billingham et al., 1966; 1963). These observations were interpreted as supporting the deletion hypothesis for self tolerance as the host was unable to prevent the donor cells from destroying the skin grafts. A very interesting experiment involving the abolition of tolerance in adult CBA mice tolerant to A type antigens by the neonatal administration of (CBAxA)F1 cells was performed by Lubaroff and Silvers (1973) Healthy A type grafts carried by CBA tolerant hosts, are rejected if C3H lymphocytes, sensitized against A type antigens, are injected into the CBA tolerant hosts. The C3H cells administered are rejected by the host as judged by two criteria. C3H skin grafts, or ^{51}Cr labelled C3H lymphocytes, injected to CBA mice that had already rejected the A grafts, were also rejected. Recovery of the host from the tolerant state takes 6-8 weeks and involves a loss of chimaerism. Recovery was assessed by the ability to reject a second type A skin graft. If normal CBAxA cells are administered after the first rejection of the graft that occurs as a result of the activity of the C3H and A cells, the second A skin graft is accepted.

Experiments by Wilson (1967,1970) established MLC reactions as an in-vitro test for tolerance by providing strong evidence for the specificity and immunological basis of this reaction. Rats injected at birth with bone marrow

cells of a different strain become tolerant as judged by the acceptance of skin grafts. Cells from these animals are specifically unable to respond in MLC reactions against cells of the tolerated strain. In addition, by the use of chromosomal markers, it was established that cells from tolerant animals are not stimulated to proliferate. Spleen cells from rats made neonatally tolerant, using the same protocol, are unable to cause GVH reactions, or runt disease when injected into appropriate hosts (Schwarz, 1968). In another set of studies rats made neonatally tolerant show a specific absense of lymphocytes able to induce GVH reactions. This lack of reactivity was not associated with the presence of either inhibitory cells or serum factors (Atkins and Ford, 1972). Experiments very suggestive of the cellular basis of transplantation tolerance were performed by Elkins. Lymphocytes from adult rats, made neonatally tolerant with allogeneic bone marrow cells, were specifically unable to mount a GVH reaction against F1 hosts. Neither lymphocytes nor serum factors from the tolerant animals were able to inhibit the MLC reactivity of normal lymphocytes in agreement with the observations already described. Male Lewis rats tolerant of LBN histocompatibility antigens and carrying healthy LBN grafts were injected with normal female Lewis cells in an attempt to break tolerance. Most of the grafts (90%) were rejected 15-25 days after cell transfer. The ability of lymphocytes from animals whose grafts were rejected to mount a GVH

reaction on appropriate male hosts was assessed and karyotypic analysis of the proliferating cells performed. Metaphases of female Lewis rats are easily distinguished as bearing 2 subacrocentric X chromosomes whereas male karyotypes have only one in addition to a distinguishable small Y chromosome. Karyotypic analysis of the cells participating in the GVH reaction showed that they were only of female origin i.e. they were derived from the donor inoculum when examined up to 5 weeks after the injection of the normal female cells. The majority of the proliferating cells examined at later times were of host male origin. In addition, thymectomy of the tolerant animals performed soon after the adoptive transfer of syngeneic female lymphocytes prevented the development of the recovery of the GVH reactivity (Elkins, 1973a, 1973b). These observations constitute some of the strongest evidence for clonal deletion. Syngeneic female cells are given to the unresponsive male hosts in sufficient numbers to obtain rejection of the tolerated skin. This number is sufficient to overcome any putative suppressor T cells or blocking factors that might be associated with the unresponsive state and hence any host precursor cells that are still functional should be induced. However, such cells were only seen 5 weeks after the injection of the female donor cells and their presence was prevented by thymectomy; this observation shows that the recovered GVH cells are newly derived from thymus dependent stem cells. For a certain period of time, 4-

5 weeks, rats that received syngeneic lymphocytes were able to reject a second LBN graft and their serum contained cytotoxic antibodies 10-28 days after cell transfer (Elkins et al., 1973). In addition, it was shown that lymphocytes, obtained from the animals 12 days after the inoculation of female cells, had low levels of GVH or MLC reactivity and were able to inhibit the ability of normal lymphocytes to mount GVH reactivity (Elkins, 1972). The origin of these suppressor cells was not identified although, from the previously described observations that no dividing cells of host origin were detected, it appears likely that they were derived from the donor inoculum.

In most of the experimental systems employing protein antigens, for inducing tolerance during embryogenesis or soon after birth, tolerance is demonstrated as a simple absence of reactivity as already described. In contrast to the many well analyzed unresponsive states induced by protein antigens in adult immunocompetent animals very few studies of a similar nature have been performed during early stages of immunocompetence.

In one system tolerance to HGG was induced in newborn mice which receive antigen through suckling from their mothers who had been previously treated with dHGG. Tolerance in this system was shown as absence of humoral immunity for a period of 18 weeks and was not associated with the presence of suppressor cells (Benjamin, 1977). In another

system a broader analysis of the tolerant state was performed in Balb/c mice. Tolerance in these animals was established in utero by transfer of antigen from pregnant mothers to the offspring after the 15th day of gestation. Tolerance was demonstrated by adoptive cell transfer experiments in irradiated syngeneic animals, at both the B and T helper cell level and was not associated with the presence of suppressor cells. Inhibitory T cells were present when tolerance was waning after 12 weeks of age; by this time the antigen had almost disappeard (Waters et al., 1979). These experiments provide evidence that tolerance induced during ontogenesis involves different mechanisms from those unresponsive states induced in adult animals. It is known, for example, that tolerance induction and maintainance to the same antigen, dHGG, may involve the presence of suppressor T cells when humoral unresponsiveness to this antigen is induced in adult animals (Doyle et al., 1976; Benjamin, 1975).

Evidence that specific B cells arising from an inoculum of fetal liver cells can be paralysed without the concomitant production of suppressor cells, was described in a different murine system. Fetal liver cells are transferred to irradiated mice and treated with deaggregated or aggregated HGG and 15 days later are rechallenged with this antigen. The donors of the fetal liver cells and the irradiated hosts are chosen so that they differ genetically in the region determining allotype. Tolerance is assessed by

the presence or absence of antibody in the serum of the treated animals. Treatment with aHGG was able to induce tolerance to B cells of fetal liver origin. In contrast, dHGG not only failed to induce tolerance but actually primed the cells. Spleen cells from tolerant animals did not inhibit the response of normal cells. Normal cells were unable to break the unresponsive state of the tolerant cells since all antibodies produced were of the host's immunoglobulin allotype (Elson, 1977).

Evidence for clonal deletion, although inferential, comes from <u>in-vitro</u> experiments where developing cells are allowed to interact with antigen and become specifically unresponsive after this interaction. Unresponsiveness is not reversed after removal of antigen and takes place in the presence of irradiated unprimed adult cells which are unlikely to include specific suppressor T cells. In detail, neonatal spleen cells or adult anti-Thy-1 treated bone marrow cells are injected to carrier (KLH or Hy) primed irradiated mice. A day later, when the donor cells have colonized the host's spleen, this organ is removed, divided in very small pieces and cultured with DNP coupled to several heterologous (non Hy or KLH) carriers such as HGG, MGG, EGG, OVA, d-GL and Lys. Pretreatment of young spleen or adult anti-Thy1 treated bone marrow cells, with DNP-carriers (with the exception of DNP-Lys) abolishes the ability of these cells to respond to DNP-protein conjugates. B cells in this system are tolerized by specific receptor-multivalent

antigen interaction unless antigen is concominantly recognized by primed T cells (Metcalf and Klinman, 1976; Metcalf et al., 1979). Similar results were obtained by Teale et al., (1979) utilizing the above described system. The difference in these studies is the characterization of the easily tolerizable cell population by means of a cell sorter which is described as a B cell present in neonatal and adult spleens and carrying low concentrations of surface immunoglobulin receptors.

The in-vitro induction of T cell tolerance has only been described once. Nevertheless, a very interesting analysis suggests that ontogenetically early MLC reactive clones can be tolerised without the concomitant production of suppressor cells. Fetal liver cells at day 15 of gestation are able to generate MLC reactive cells after 4 days of organ culture in the presence of adult syngeneic cells. Culture with adult allogeneic cells led to specific inhibition of the appearance of MLR reactive lymphocytes. This type of tolerance was not associated with the presence of suppressor cells, as shown by the inability of these cells to inhibit the response of normal adult lymphocytes (Robinson and Owen, 1978). Finally a particular form of deletion of self reactive clones has been suggested recently. According to this view, lymphocytes having receptors for transplantation antigens are deleted when they encounter antigen on the surface of a particular infrequent cell type, present in the thymus of normal mice and in the

spleens of nude mice. This cell is able to provide a lethal signal to the responding cell (Miller and Derry, 1979).

B. Active inhibition by specific serum factors

Voisin et al (1968, 1972) were the first to prospose that anti-self reactivity is regulated by an active immune response. Immunity to antigens present on living cells is usually of the cell-mediated class which is the response that can effectively destroy them. To ensure the "neutralization" of cellular immunity the animal is supposed to produce a class of humoral response against the target antigens. The non-cytotoxic antibodies that are formed cover the antigens and prevent their recognition by lymphocytes that mount cell-mediated responses. Voisin called these antibodies "synergic" or "facilitating" and considered them as a class of non-complement fixing antibodies coexisting with both a depressed cellular response and depressed production of complement fixing antibodies. He supported his views by demonstrating the existence of these "facilitating" antibodies in the sera of CBA mice which had been rendered neonatally tolerant with (CBAXA) cells and were carrying long term healthy A type grafts. Sera from these animals did not contain direct haemaglutinating antibodies against MHC antigens of the A strain. Their presence could be revealed by a synergistic effect involving an increase in the haemaglutinating titer of a standard anti-A serum. Furthermore he claimed that sera from tolerant mice were able to enhance the growth of donor type tumours grafted in

normal mice.

The _in-vivo_ role of antibodies present in allograft bearing animals is not clear. Efforts to reproduce the previously described observations have been unsuccessful. Serum from mice, made neonatally tolerant to MHC antigens, failed to enhance the growth of tumors in normal mice, although hyperimmune sera which contained cytotoxic antibodies were able to inhibit the _in-vitro_ killing of target cells by effector lymphocytes as well as being able to enhance the growth of tumors in normal mice (Law et al., 1974). There are, however, several reports of antibodies being present in experimental systems where tolerance has been induced neonatally in mice and rats. These antibodies are detected by their ability to specifically inhibit the _in-vitro_ response of normal lymphocytes against target cells of donor type. I. Hellstrom et al., (1969) observed that lymphocytes from tumor bearing animals are able to inhibit the growth of tumor cells _in-vitro_ but sera from these same animals can block this activity. They also extended their observations to the question of why mothers do not reject their fetuses. They showed that lymphocytes from allogeneically pregnant mice are able to inhibit the growth of fetal cells _in-vitro_ and that maternal serum could block this inhibition (Hellstrom et al., 1969). These observations led to the suggestion that neonatally induced tolerance to living cells and the lack of an effective response by a mother, against fetuses she is carrying, might have a

similar basis. CBA mice injected at birth with (CBA x A)F1 spleen cells, and carrying A skin grafts as adults, contained lymphocytes able to give small but significant killing of lung type A fibroblasts in a 2 day in-vitro assay. This activity was inhibited by serum from tolerant animals (I. Hellstrom et al., 1971). The same observations were repeated in rats. These animals, made neonatally tolerant, were able to carry healthy donor type grafts as adults. Their lymphocytes did not have any detectable MLC reactivity but they contained a class of effector lymphocytes able to kill lung fibroblasts of the donor type in-vitro and their sera could inhibit the killing activity (Wright et al., 1975). The most important argument for the existence of humoral blocking factors in an experimental situation that mimics self tolerance comes from studies with tetraparental mice. Lymphocytes from such animals were able to kill parental cell types in-vitro and their sera could prevent this killing (Wegmann et al., 1971). Furthermore, serum or non-T cells from these mice were able to inhibit the MLC reactivity of normal lymphocytes. It was noticed in these experiments that none of the sera, or non-T cells tested, displayed the same level of inhibitory activity and there was suggestive evidence that the antibodies found were directed against MHC antigens, without being cytotoxic in the presence of complement (Phillips et al., 1971; Phillips and Wegmann, 1973).

There are two important points to be made with regard

to these observations. First, there is an important difference between the mechanisms proposed by Voisin et al., (1968,1972) and that emerging from the experiments of Hellstrom et al., (1971), Wegmann et al., (1971) and Wright et al., (1975). In the first case the induction of cell mediated immunity is blocked by a particular class of humoral immunity whereas in the second case effector cytotoxic cells are demonstrated as continuously present. It is difficult to imagine how cell mediated effector lymphocytes existing <u>in-vivo</u> are prevented indefinitely from causing damage. It seems more reasonable that as proposed by Voisin, humoral immunity prevents the induction of cytotoxic cells rather than continuously blocking their effector function. Support for this view comes from the observations of Mintz and Silvers (1967). They reported that about 700 chimaeric mice, observed over a period of 3 years, did not display any signs of runting syndrome. Perhaps these observations can be reconciled if the killer lymphocytes in the microcytotoxicity assay are an expression of humoral rather than cell-mediated immunity. Indeed it appears that the cell involved in the killing in this assay is a null cell (a cell without B or T cell markers). As little as one B-cell producing antibody per culture is sufficient for killing to be detected (Blair and Lane, 1975).

Antibodies directed against the MHC antigens are known to be able to specifically inhibit MLR responses (Schwartz et al., 1978). It therefore is not suprising that antibodies

present in allograft tolerant animals can sometimes inhibit the induction of MLR reactivity. The failure by different investigators to inhibit the GVH reactivity of normal cells with sera from tolerant animals has been considered as an argument against the physiological importance or even the existence of blocking antibodies. This argument is misleading. It appears that the blocking by serum of in-vitro killing is due to molecules with specificity for the target cell, whereas blocking of GVH reactivity is usually due to molecules specific for receptors on donor cells able to recognize the host. The two assay systems for blocking factors, present in the serum of unresponsive animals, are measuring the activity of two quite different molecules.

The second major question is whether tetraparental animals are a valid model for self tolerance as normal F1 animals do not display similar activities as cells and serum from allophenic mice. Not all allophenic mice behave the same way and differently from F1 mice as described earlier (Barnes and Graham, 1976). It seems that tetraparental mice should be a valid model for studying the basis of self tolerance if it is known that their immune system remains chimaeric throughout their life.

The possible existence of antibodies against receptors on lymphocytes with specificity for self antigens gives another dimension to the regulation of the antiself immune response by humoral antibodies, as described in an earlier

chapter. Induction of anti-receptor antibody would a priori require an active anti-self response preceding the induction of tolerance. This immunity, even at low levels, should be continuously present in order to stimulate the anti-idiotypic response.

Experimentally, anti-receptor antibodies with specificity for MHC alloantigens are usually produced in hybrid animals upon immunization with parental type lymphoid cells. Injection of CBA spleen cells into (CBA x A)F1 animals leads to the formation of two serum activities. First, F1 animals respond to the idiotype of the receptor molecules specific for alloantigen A, present on parental type CBA cells. This antibody is called anti receptor antibody and is the first serum activity detected in the immunized animals. With more prolonged immunization alloantibodies made by CBA cells against A antigens are detected in the serum (Ramseier, 1974; Ramseier and Lindenmann, 1975).

Ramseier (1973) observed that CBA mice, made neonatally tolerant by administering adult (CBA x A)F1 spleen cells and able to carry for long periods healthy A type grafts, do not reject the grafts even they are injected with large numbers of adult syngeneic CBA lymphocytes. Furthermore, larger numbers of CBA lymphocytes, that are effective in breaking tolerance, were unable to do so when the tolerant mice had been pre-injected with small numbers of CBA lymphocytes.

After the cell transfer, these animals were shown to contain in their sera anti-receptor antibodies. The titers of these antibodies diminished with time and concomitantly the titers of anti-A alloantibodies increased. The author attributed the inability of normal cells to break the unresponsive state to the existence in the animals of low levels of anti-receptor antibodies produced by cells of the F1 host. Such antibodies were postulated to inactivate the donor CBA lymphocytes so that they were unable to destroy the A graft. The interpretation offered by Ramseier assigns a functional role to the serum antibodies found in some of the allograft tolerant animals. In contrast to blocking factors that are alloantibodies, or antigen-antibody complexes, anti receptor or anti-idiotypic antibodies have been shown to exert <u>in-vivo</u> and <u>in-vitro</u> activity. They have been shown to inhibit the ability of lymphocytes to mount a lethal GVH reaction in mice (Joller, 1972) or a local GVH reaction in rats (Binz et al., 1973) and in mice (Binz, 1975). Also, anti-receptor antibodies were able to inhibit the effector cytotoxic cells in killing their target cells (Kimura, 1974) or inhibit MLC reactive cells (Binz and Askonas, 1975). Finally, anti-receptor antibodies administered to adult rats were able to induce specific transplantation tolerance in these animals (Binz and Wigzell, 1976). It should be mentioned that anti-idiotypic antibodies are not always effective in inhibiting cell-mediated immune functions (Lindahl, 1972; Fitch and Ramseier, 1976) and in some cases have shown to be able to

either suppress (Eichmann, 1974) or specifically stimulate an immune response (Eichmann and Rajewsky, 1975).

The presence of a serum blocking factor with functional similarity to that of an anti-receptor antibody was described in chickens made embryonically tolerant with allogeneic embryonic spleen cells. Serum from these birds, which were carrying long lasting healthy donor type grafts, was able to inhibit the ability of normal lymphocytes to mount a GVH splenomegaly reaction (Droege and Mayor, 1975). The interpretation of these experiments, however, is not straight forward. The strains used are not fully inbred. It is possible that the blocking antibodies found are directed against non B antigens present on the skin grafts. It is well known that the GVH splenomegaly reaction is effectively inhibited with low concentrations of antibodies when they are directed against antigenic structures present on lymphocytes (Fredericksen et al., 1977).

C. Active inhibition by specific T cells

Evidence has accumulated in recent years that T cells from unresponsive animals are able to specifically inhibit the immune responsiveness of normal lymphocytes both in-vivo and in-vitro. Historically, the first evidence for an active element maintaining the unresponsive state in tolerant animals came from the experiments of McGregor, McCullagh and Gowans (1967). They showed that the administration to irradiated syngeneic rats of 2.5 x 10^8 lymphocytes from

rats, made neonatally tolerant to SRBC, were unable to restore the anti SRBC response of the recipients whereas the same number of normal cells could. When the same number of unresponsive cells were incubated for 12 hours at 37°C, before being injected into the irradiated recipients, the response was restored. This finding implied the existence of viable precursor cells in the unresponsive population. Subsequently, McCullagh (1970a) observed that injection of normal lymphocytes into sublethally irradiated rats, made tolerant to SRBC by the same procedure as in the experiments already described, could not restore the antibody response although the same number of cells administered to normal irradiated animals could. This finding strongly suggested the presence of a factor in the tolerant animals capable of inhibiting the response of normal lymphocytes. Furthermore, it was shown that antibody precursor cells were present in the spleens of tolerant animals for a period of 3 days after the transfer of normal cells although no direct PFCs were ever detected in these populations. Following this 3 day period, precursor cells with specificity for SRBC disappeared from the spleens of the tolerant hosts (McCullagh, 1970b). The existence of an inhibitory element in the spleen of mice made neonatally tolerant to SRBC was demonstrated as an ability of this population to specifically inhibit the cooperation of B and T lymphocytes from normal animals to produce an anti-SRBC antibody response. These experiments also provided evidence that the

inhibitory element was a thymus dependent cell, since spleen cells from tolerant, thymectomized mice were not able to inhibit the immune response (Gershon and Kondo, 1971). Finally, at the same period, Droege (1971) reported that two types of cell exist in the thymus of chickens, one showing helper activity by its ability to cooperate with B cells in an antibody response, the other being bursa-dependent and displaying an ability to inhibit the antibody response.

The role and mode of action of suppressor T cells as regulators of anti-self immune reactivity is unclear. As described in the last chapter there is a strong argument for their presence if autoimmune responses occur at a low level without leading to deleterious autoimmunity (Cunningham, 1976). Indirect evidence for suppressor cells regulating tolerance to self antigens comes from observations in which neonatal animals harbour T cells able to inhibit the immune response of normal adult lymphocytes in a non-specific fashion. The argument behind these experiments is that, should suppressor cells be responsible for tolerance induction to self antigens, their presence should be particularly evident around the neonatal period during which animals are particularly sensitive to tolerance induction. For example, fetal mouse liver cells are able to inhibit the ability of adult syngeneic or allogeneic lymphocytes to generate MLC or GVH reactive cells (Globerson and Umiel, 1978). Spleen cells from newborn mice can inhibit non specifically the antibody response of adult B cells (Mosier

et al., 1975). A class of thymic, bursa-dependent lymphocyte, present in young chickens, is capable on transfer to adult sublethally irradiated syngeneic or allogeneic chickens of suppressing the ability of these birds to reject allogeneic grafts, to mount GVH reactions and to produce an antibody response (Droege, 1976).

Tolerance to MHC alloantigens, induced at an early developmental stage by injection of living lymphoid cells, has long been considered a model for the induction and maintainance of tolerance to self antigens. The number of reports on the positive demonstration of specific suppressor cells in these systems is rather limited and can be divided into two kinds. In the first category the evidence is either indirect or the inhibitory activity, in order to be revealed, requires the transfer of tolerant lymphocytes to irradiated or sublethally irradiated hosts for a long period. Experimental systems of this nature are the following. Mice that have been rendered neonatally tolerant to MHC alloantigens and carry healthy long lasting grafts are able to reject the grafts when the axillary and branchial nodes are removed. When such lymph node cells are reinjected to these mice they are able to accept a second graft (Silvers, 1974). Lymphocytes from adult rats, made neonatally tolerant to MHC alloantigens, are able to specifically prolong the acceptance time of grafts upon transfer to sublethally irradiated syngeneic recipients. It seems in these experiments that irradiation of the hosts is

essential for the detection of these suppressor cells as a mixture of the suppressor containing population with normal lymphocytes, even at a ratio of 10:1, cannot inhibit the ability of the normal lymphocytes to reject the grafts (Dorsch and Roser, 1975; 1977). A long period of in-vitro culture, or transfer to irradiated hosts, is required to reveal suppressor cell activity in another similar system developed in mice. Lymphocytes from mice made neonatally tolerant to MHC alloantigens are cultured for 5 days with antigen and then tested for their ability to inhibit the 5 day in-vitro induction of cytotoxic T cells mounted by normal cells. The inhibition observed in this system is believed to be exerted by a suppressor T cell that inhibits the generation of killer cells from cytotoxic T cell precursors. During the same in-vitro culture period a second type of suppressor cell is produced which is capable of inhibiting the generation of killer precursor cells from stem cells. This cell is physically seperable from the previous one and reveals its inhibitory activity after transfer with normal syngeneic bone marrow cells to irradiated hosts for a period of 50 days. Cells taken from the reconstituted mice not only produce low levels of cytotoxic T cell responses when challenged in-vitro but could specifically inhibit the cytotoxic response of normal syngeneic cells. Interestingly enough this second class of suppressor T cell is also able to specifically inhibit the generation of killer cells when given to newborn mice in the

absence of antigen. This property suggests that their specificity is directed against the idiotype present on the receptor of the cytotoxic precursor that has specificity for the alloantigen (Gorczynski et al., 1978; Gorczynski and MacRae, 1979a; 1979b).

Direct evidence for the existence of specific suppressor cells in the spleens of adult mice, made neonatally tolerant to MHC alloantigens, is provided in the experimental system described by Holau et al., (1978). Lymphocytes from tolerant mice which are unable to generate cytotoxic cells in-vitro can do so when adherent cells are removed by passing the cells through a nylon wool column. The adherent population does not produce a cytotoxic T cell response itself but, on the contrary, can inhibit the cytotoxic response of normal lymphocytes. Suppressor cells existing in the peripheral blood of chickens, made embryonically tolerant to several alloantigens, have been described by Rouse and Warner (1975). It is difficult to evaluate the significance of these experiments as outbred populations of chickens were used.

The work described in this introduction has not led to an agreement on the nature of the cellular basis of self tolerance. Most of those interested in the subject support either a deletion or an inhibitory mechanism for preventing the induction of anti-self reactivity. These two mechanisms however are not exclusive of one another as an inhibitory

mechanism can lead to the deletion of anti-self precursors. Originally, clonal deletion was proposed and favored. As more experimental systems of specific unresponsiveness were developed, demonstrating the existence of inhibitory T cells and/or blocking factors together with inhibited but functional precursor cells, the deletion hypothesis came under serious challenge. While the cellular nature of self tolerance is unclear, it is generally accepted that certain conditions must be met in order for an animal to be tolerant of self antigens. These important conditions have been discussed extensively above. They are that self antigens be present at high concentration both early in development before the immune system has developed and continously thereafter throughout the life of the individual. The work in this thesis attempts to clarify what kinds of unresponsive states might be analogous to self tolerance by systematically varying these most important parameters for inducing unresponsiveness to transplantation antigens and examining the biological characteristics of the different kinds of unresponsiveness obtained.

7. Introduction to Research Project

The purpose of the work presented in this thesis is to obtain an experimental system of unresponsiveness that can be used to study the cellular basis of tolerance to self antigens. From the information presented in the introductory section it is evident that there are two fundamental properties that such a system should possess: i) the antigen should be introduced early in the ontogenic development of the immune system before the lymphopoetic tissue develops immunocompetence, and ii) the antigen concentration should be maintained at a high level through ontogeny and the post-natal life of the individual. Natural unresponsiveness to the majority of self antigens is generally complete in that it covers all classes of immunity. It is therefore important to establish if the type of unresponsiveness induced experimentally extends to different classes of immunity.

The experimental system developed is the induction of specific unresponsiveness to MHC alloantigens in chickens and possesses these two properties. The antigen, in the form of embryonic erythropoetic stem cells, is introduced to allogeneic chicken embryos at early stages of embryogenesis. The self renewal ability of the stem cells ensures the continuous presence of the antigen in high concentrations for the life span of the birds. Analysis of the immunological properties of these permanantly chimaeric

healthy birds should provide valid information concerning the cellular basis of the unresponsive state and hence of self tolerance.

This avian system provides one not only with the unique opportunity of establishing a valid model for self tolerance but also the means of analyzing its cellular basis. Reagents have been developed that allow one to reliably ascertain the degree of erythroid chimaerism of the adult birds at any stage of the experimental analysis. In addition, alloreactivity can be assessed at two different levels of immune reactivity. Cell mediated immunity is mainly studied by a GVH reaction, namely the CAM-POCK assay, which is a sensitive assay essentially enumerating single alloreactive lymphocytes. Humoral immunity is analyzed either with a direct or indirect red cell agglutination assay. The indirect assay has been shown recently to reveal high titers of natural antibodies directed against the MHC encoded alloantigens of the species with the exception of self antigens in the sera of normal adult birds, (Longenecker et al., 1979). The ease with which stem cells can be injected to chicken embryos at different stages of embryonic development allowed two additional but relevant studies to be made. i) It was possible to determine the relationship between the time during the development of the immune system at which antigen is administered and chimaerism established, and the degree and type of unresponsiveness observed. This type of analysis led to the finding that there are dramatic

differences in the immunological status of the host birds when chimaerism is established at different stages of embryogenesis. ii) It was possible to study the immunological state of birds, that had been injected with allogeneic stem cells during ontogeny, at hatching, which is the earliest time that cell-mediated immunocompetence can be detected in the spleens of normal birds as measured by GVH reactivity.

II. Materials and Methods

1. Animals

SC and FP fertilised eggs were purchased from Hy-Line International, Johnston, Iowa, USA. These eggs are hybrids between different inbred strains of white Leghorn chickens. The SC and FP birds carry the B^{2}/B^{2} and B^{15}/B^{21} MHC alleles respectively. All chicks were hatched and raised in the facilities of the University of Alberta. Fertile eggs from randomly bred white Leghorn chickens were obtained from the U of A poultry research farm. Although the frequency of the MHC coded alloantigens has not been analyzed in this outbred population, it should be mentioned that in chickens, in contrast to mouse and man, common alleles are easily identified among unrelated populations (Pazderka et al., 1975). When available, fertile eggs from B^{14}/B^{14}, B^{15}/B^{15}, B^{19}/B^{19}, B^{13}/B^{13}, and B^{21}/B^{21} strains were used. These strains are maintained in the facilities of U of A. The histocompatibility type of these birds has been determined serologically. In addition, the GVH CAM-POCK test has been used to confirm the serological identification for these strains (Pazderka et al., 1975, B.M. Longenecker, pers. comm.).

2. Production of Chimaeras

Two different methods for generating haematopoietic chimaeras using the SC and FP strains were employed in this study.

(A) Parabiosis: Two 12-day-old embryos of the two different known genotypes (SC and FP) are joined together through their chorioallantoic membranes and sealed with paraffin wax.

At day 12 of incubation, windows are marked on the midpoint of the long axis (as described later for splenomegaly) over a forked vein. The eggshell and the shell membranes are then removed so that the chorioallantoic membrane is fully uncovered. Immediately thereafter, the two genetically different eggs are joined together by their exposed CAM's using paraffin wax, and then returned to the incubator. Care is taken to turn the parabionts at least once daily. Close to the time of hatching, the parabionts are put in nylon stockings and separared from each other with masking tape, in such a way that each chick will hatch in different compartments of the nylon stocking. This allows the birds to remain identified with respect to genotype, (Fig 1, A).

(B) Stem cell transfer: The source of the haematopoietic stem cells is the embryonic spleen.

Routinely, 30 pooled spleens of 15, 17 or 21 day-old SC or FP embryos are used as the donor cell population. Spleens are minced carefully in 10 ml of RPMI-1640 serum free medium

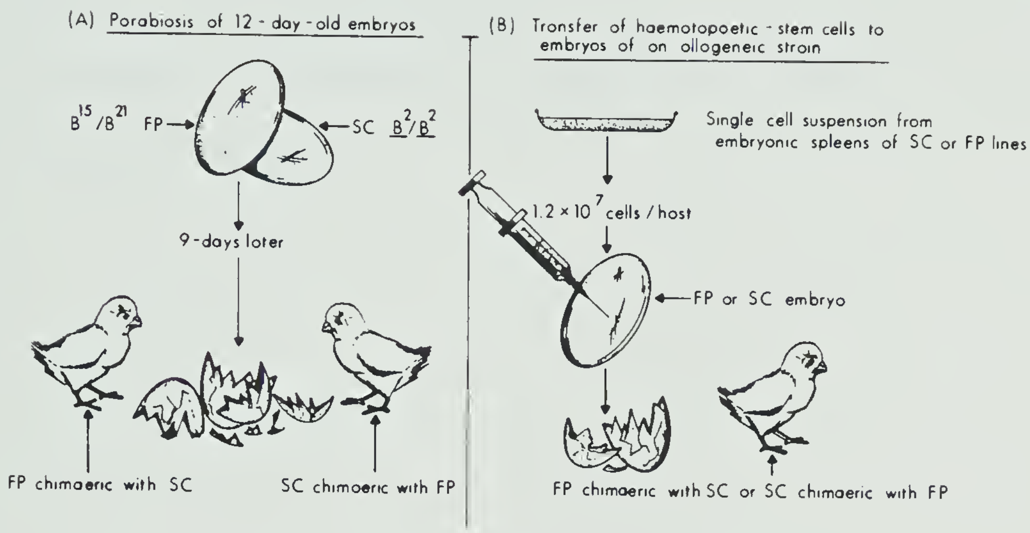

Fig 1. Production of chimaeric chickens. In A, two 12 day-old embryos of FP and SC strains are joined together to obtain either FP birds chimaeric with SC or SC birds chimaeric with FP. In B, a single cell suspension containing stem cells is injected into allogeneic embryos. Details of the preparation of donor cells are described in Materials and Methods. In most cases the age of the donor and the recipient were matched. Chimaeras were made at days 15, 17 or 21 of embroygenesis by injecting stem cells intravenously (on day 15 and 17) or intraperitoneally (on day 21). A fourth type of chimaera was generated by injecting stem cells from 15 day old embryos to 21 day old allogeneic recipients.

until single cell suspensions are obtained.
The cells are then allowed to settle for 20 minutes through 10 mls of medium in 12 ml test tubes. The supernatants, which contain the haematopoetic stem cells (Keller et al., 1979), are harvested. The cell concentration is adjusted so that 1.2×10^7 non-red spleen cells are injected intravenously into 15-day-old SC or FP embryos. The same number of 17 or 21 day old spleen cells were in some experiments also administered either intravenously to 17 day old SC embryos or intraperitoneally to newly hatched SC chicks. (Fig 1, B).

3. Production of anti-B sera

Specific alloantisera were produced by reciprocal immunizations between strains SC and FP. Each bird received 3 injections each of 2 ml of citrated adult whole blood every second day. 6 days after the last injection the haemagglutination titer was determined. If the titer was lower than 1:32 the above immunization procedure was repeated after a 2 week rest period. As the strains used are not inbred for all non-B loci the hyperimmune sera produced were extensively absorbed with erythrocytes from many different members of the host strain. Absorption was carried out at room temparature for 30 minutes using equal volumes of antiserum and packed RBCs. The procedure was repeated 3 times. Finally, the serum was separated in small volumes and stored at -30°C.

4. Detection of Chimaerism

All birds generated by the two methods described previously were tested for red blood cell chimaerism using the haplotype specific anti-B locus sera, produced as described above. Two methods were employed for determining the extend of chimaerism.

A. Red blood cell agglutination

Red cell agglutination was the assay routinely used for a qualitative and semi-quantitative estimate of chimaerism. Before typing the putative chimaeras, the strength of the antiserum was characterized by typing different mixtures of RBCs from the two strains used. Generally, a 10% chimaerism was easily detected and a strong antiserum could often detect as little as 5% chimaerism.

B. Cellular radioimmunoassay

Details on this assay have been published recently (Longenecker et al., 1978). Briefly, 10^7 red cells from the putative chimaeras were incubated with 0.1 ml of specific alloantiserum for 30 minutes at R.T. Then the cells were washed four times and further incubated with 0.2 ml of ^{125}I-rabbit anti-chicken immunoglobulin. After incubation for 30 minutes at room temparature, the washing procedure was repeated and the cells were harvested and counted. The degree of chimaerism was estimated by comparing the counts found with those from artificial mixtures.

5. <u>GVH Reactions</u>

Cell mediated responsiveness was analyzed by two manifestations of GVH reactivity, namely the splenomegaly and the CAM-POCK reactions.

A. Splenomegaly: The reaction involves spleen enlargement as measured by increment in weight which results from the intravenous (i.v.) injection of immunocompetent lymphocytes into 12-day-old embryos measured 4 days post-injection. The assay is known to measure alloreactivity due to major B complex differences (Schierman and Nordskog, 1963).

B. CAM-POCK reaction: This involves the formation of pocks (lesions of characteristic morphology and size) following inoculation of donor immunocompetent lymphocytes onto the chorioallantoic membrane of 12-day-old embryos differing from the donor lymphocytes at the B locus. Pocks are enumerated 4 days post-inoculation.

It should be emphasized that the time at which spleens are weighed or pocks are counted in the above assays is important. It has been shown that the day 4 spleen weight or day 4 pocks are due to reactivity against antigens of the major B histocompatibility region (Longenecker et al., 1970). Any further enlargement of the spleen, or increment in number and quality of pocks at day 5 or 6 are due to

minor histocompatibility loci differences (Longenecker et al., 1973). Since the strains used above are only inbred with respect to the B region it is important to assay at day 4 to avoid complications due to minor differences.

C. Donor cell preparation

a) Peripheral blood lymphocytes (PBLs): Heparinized (10 units/ml) peripheral blood is obtained from appropriate donors. The blood is then distributed in 5 ml culture plastic tubes (4 ml/tube) and centrifuged at 600 rpm for 6 min. The lymphocyte-rich plasma supernatant is collected while avoiding any red blood cell contamination. (If the yield of cells is insufficient, a second centrifugation at 400 rpm for 3 min. is employed. This will give another layer of lymphocyte-rich plasma. The lymphocytes are washed twice in RPMI-1640 by centrifugation at 1200 rpm for 6 min. Finally, the cells are resuspended and viable counts are made using trypan blue dye.

b) Spleen cells: Donor spleens are removed under sterile conditions and minced in RPMI-1640 until a single cell suspension is obtained. Cell clumps are allowed to settle out for 15 min. under gravity in 15 ml culture plastic tubes (about 10 ml/tube) and the single cell containing supernatant is collected. The cells are washed twice and counted as before.

D. Assays

a) Splenomegaly: The eggs used as hosts for splenomegaly are incubated for 12 days. With the aid of a candle lamp, a

small rectangle is marked around a large CAM vein and this area is disinfected with 25% iodine in 70% alcohol. By means of a Dremel tool (electric circular drill), the rectangular window is opened by cutting carefully (without damaging the shell membranes). The edges of the marked rectangle and the little square eggshell is removed with the aid of a sharp forceps.

A drop of paraffin oil makes the shell membranes transparent, revealing the vein underneath for injection with a tuberculin syringe. 0.1 ml of cell suspension is injected per embryo and the exposed shell membranes are sealed with paraffin wax. For each group, 9 to 12 embryos are used as recipients.

4 days later, the eggs are opened and spleens are removed and weighed. Direct spleen weight, compared to that of B-matched injected embryos, represents the magnitude of the GVH.

b) CAM-POCKS: The eggs used as hosts for CAM-POCKS are incubated for 12 days and candled for fertility as before at the day of experimentation.

The assay requires direct inoculation of lymphocytes onto the dropped chorioallantoic membrane (CAM). The egghsell is punctured in two places. The first puncture is made at the tip of the air sac. The second one is made on the uppermost side, midway along the long axis while the egg is held with its long axis in a horizontal position. The second puncture is a small slit carefully made to avoid touching the CAM.

After these two punctures are made, the CAM is dropped with the aid of a gentle vacuum applied through the first puncture in order to produce an air space under the second puncture. Then the egg is rotated about 90° on its long axis, while held horizontally. This brings the air sac under an undamaged part of the shell. The top of the air space is marked and a window is opened with the aid of the Dremel tool. The opening is covered temporarily with masking tape until the time of inoculation. 0.1 ml of cell suspension is inoculated on the top of the CAM by the use of a 0.1 ml pipette. Finally, the openings are sealed with the masking tape and paraffin wax. Generally, 9 to 12 embryos are used as recipients per experimental group. 4 days later, the eggs are opened and the inoculated parts of the CAMs are collected, washed and kept in saline until counts are made under a stereoscopic microscope.

E. Haemagglutination assays

a) Direct haemagglutination

0.1 ml of sera were serially diluted two-fold in PBS in U-bottom microtiter plates and 0.1 ml of 0.5% red cells were added to each well. The plates were incubated for 45 minutes at R.T. and red cell agglutination was directly read through a mirror in order to better visualise the haemagglutination.

b) Indirect haemagglutination

0.1 ml of sera were serially diluted two-fold in PBS in V-bottom microtiter plates and 0.1 ml of 0.5% red cells were added to each well. Plates were incubated for 45 min and washed twice with 0.2 ml of PBS. The cells were then transferred to new U-bottom plates and 0.1 ml of developing horse anti chicken immunoglobulin serum was added. Transfer to new plates was necessary as chicken immunoglobulin sticks to the plastic wells and causes misleading agglutination of the red cells on the addition of rabbit anti-chicken immunoglobulin. 45 minutes later red cell agglutination was directly read through a mirror. The above reactions are all carried out at room temperature.

III. Results

In this study haematopoietic avian chimaeras have been produced by administering erythropoietic stem cells to allogeneic chicken embryos at different stages of embryogenesis. The results show that the immunological and pathological characteristics of these chimaeras differ dramatically depending on the stage of ontogenic development at which they are generated. The description of the results is therefore subdivided into three sections, each describing the immunological and/or pathological properties of chimaeras generated at different stages of embryonic development.

1. Chimaeras generated at days 12 and 15 of embryonic development

A. Establishment and estimation of chimaerism

Chimaerism was established at day 12 of embryogenesis by parabiosis between two embryos of strains SC and FP as described in Materials and Methods (Fig. 1. A). Both types of chimaeras, that is SC birds chimaeric with FP, and FP birds chimaeric with SC red cells, were produced. Parabiosis at day 12 of embryogenesis allows recirculation of stem cells from one embryo to the other for about 9 days. This continuous flow of stem cells results in adult birds displaying high levels of chimaerism for their entire life time. This method of producing chimaeras is hampered by the

limited number of parabionts one can produce in a given amount of time. In addition there is a high mortality rate in the first 24 hours after the operation. It is a common finding that one of the developing chickens of a pair, although surviving the joining procedure, dies 3 or 4 days later. When care is taken that this dead partner is removed before intoxicating the live embryo, a healthy chick is hatched that is usually chimaeric. Sometimes both partners survive; in all such cases both birds were found to be chimaeric. A total of 8 SC and 9 FP chimaeric birds were produced. These birds were kindly typed by Dr. Pazderka between 4 and 5 weeks of age. Through the course of these studies the same birds were retyped and stable chimaerism was found to persist over a period of 4 years. Table 1, shows an experiment in which putative chimaeras were typed by the red cell agglutination assay and demonstrates that all 7 birds tested for erythroid chimaerism at 120 weeks of age were still chimaeric.

Successful chimaerism was induced at day 15 of embryogenesis by intravenous administration of embryonic stem cells into allogeneic embryos as described in Materials and Methods (Fig. 1. B). The embryonic chicken spleen has been shown to contain a non adherent, non aggregating cell population which contains the erythroid stem cells. These stem cells are easily identified in the spleens of 15 day old embryos, reach peak numbers at day 17 and then decline (Keller et al, 1979). This stem cell fraction is obtained if

Table 1. Semiquantitative determination of chimaerism by red blood cell agglutination

Source of RBCs	Dilution of:											
Artificial Mix FP/SC Ratio	anti-FP serum					anti-SC serum					Percentage of Chimaerism	
	1/1,	1/2,	1/4,	1/8,	1/16	1/1,	1/2,	1/4,	1/8,	1/16	FP	SC
0/100	O	O	O	O	O	+++	+++	+++	+++	+++	0	100
10/90	+	O	O	O	O	+++	+++	+++	+++	+++	10	X
20/80	+++	+	O	O	O	+++	+++	+++	+++	+++	20	X
30/70	+++	++	+	O	O	+++	+++	+++	+++	+++	30	X
40/60	+++	+++	++	+	O	+++	+++	+++	+++	+++	40	X
50/50	+++	+++	+++	++	+	+++	+++	+++	+++	+	50	50
60/40	+++	+++	+++	+++	+++	+++	+++	++	+	O	60	40
70/30	+++	+++	+++	+++	+++	+++	++	+	O	O	X	30
80/20	+++	+++	+++	+++	+++	++	+	O	O	O	X	20
90/10	+++	+++	+++	+++	+++	+	O	O	O	O	X	10
100/0	+++	+++	+++	+++	+++	O	O	O	O	O	100	0
FP												
#1						+++	+++	++	+	O	40	
#2						+	O	O	O	O	10	
#3						+++	+	O	O	O	20	
#4						+++	+++	++	+	O	40	
#5						+	O	O	O	O	10	
SC*												
#1	+++	+++	+++	++	+						40-50	
#2	+++	+++	++	+	+						40	

SC* and FP* 90 week old birds, made chimaeric at day 12 of embryogenesis. The agglutination was carried out as described in Materials and Methods.

+++ = complete agglutination
++ = partial agglutination
+ = small but definite agglutination
O = no visible agglutination
X = not possible to estimate

the single cell suspension of embryonic spleen is allowed to settle for 20 minutes and the supernate is collected. The successful production of red cell chimaeras proves that the embryonic spleen contains erythroid stem cells. Intravenous administration of 15 day old embryonic spleen cells to 15 day old allogeneic embryos allows the production of large numbers of putative chimaeras in a short period of time. Large numbers of both types of chimaeras were generated using this method. The immunological characteristics of FP birds chimaeric with SC red cells, hereafter, referred to as FP* were identical with those of SC birds chimaeric with FP (SC*). Therefore some of the studies carried out after the 2nd year of this research included only SC* birds. A total of 91 SC* and 29 FP* birds were analyzed for several different parameters individually; all of these birds were chimaeric. Fig. 2 shows the results of typing SC* birds made at day 15 of embryogenesis. All 13 birds of this group were shown to be chimaeric. The degree of success in generating chimaeras in 3 different groups was 100%, (Fig. 2), 85% and 80% (results not shown). A bird judged to be chimaeric had at least 5% of donor type red cells. The earliest time at which it was possible to detect chimaerism was the third week after hatching. This rather late time may reflect the fact that tests for chimaerism utilised recirculating blood cells; 3 weeks may be required for stem cells to mature and give rise to red cells in the circulation. It does not reflect the late appearance of B antigens as the same

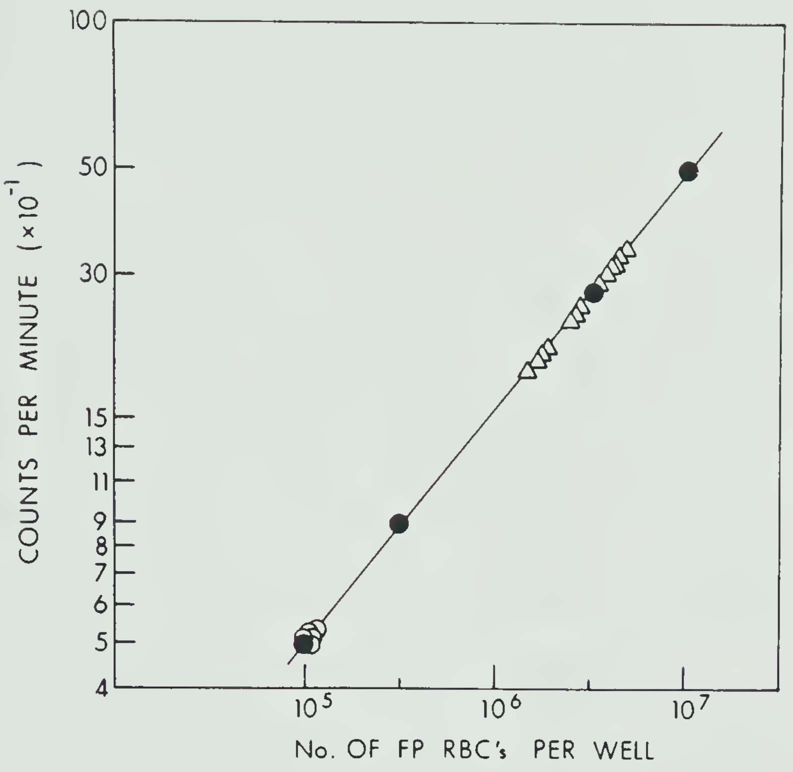

Fig. 2. Quantitative determination of chimaerism by cellular radioimmunoassay. Open triangles represent the number of counts from 13, 8 week old SC* birds made chimaeric at day 15 of embryogenesis. Closed circles represent the standard curve generated by reacting artificial mixes of SC and FP red cells with anti-FP serum. A total of 10^7 red cells were used per well. Open circles represent the number of counts from 5 control SC birds following incubation of 10^7 red cells with anti-FP serum.

reagents used to type the adult chimaeric birds are able to detect specific alloantigens on 15-day-old embryonic spleen cells.

B. Immunological and Pathological Properties

a. Cell Mediated Immunity.

i) Specific absence of GVH alloreactive clones in adult chicken chimaeras generated at days 12 and 15 of embryogenesis.

Two manifestations of GVH reaction were employed to assess cell-mediated immunity. These are GVH splenomegaly and the CAM-POCK reaction described in Materials and Methods. Both types of reactions are assayed 4 days after administring the cells and they are a measure of alloreactivity due to antigenic disparity at the MHC B region. All experimental birds used in these studies have been typed positively as erythroid chimaeras.

Table 2, shows the results obtained when lymphocytes from 8 FP* birds were tested individually for their ability to cause GVH splenomegaly reactions. In exp #1, lymphocytes from 3 14 week old chimaeric birds, generated at day 12 of embryogenesis by parabiosis, were tested for their ability to cause splenomegaly when injected into embryos of the donor type strain or into third party embryos. In exp. #2, lymphocytes from 5 FP* birds made chimaeric at day 15 of embryogenesis, are also examined for their ability to cause splenomegaly. As can be seen complete tolerance was induced

Table 2. Graft-Versus-Host reactivity of adult chimaeric birds, generated at days 12 and 15 of embryogenesis, as assayed by splenomegaly[1]

Source of donor cells	Weight of spleens (in mgs) 4 days post injection: SC Hosts[5] (mean±SE)	Outbred Hosts[5] (mean±SE)
exp #1		
FP*[2] #1	11.97±0.38(i)	78.5±14.6
FP*[2] #2	12.4 ±0.6 (i)	58.8±13.4
FP*[2] #3	11.25±0.4 (i)	81.2±16.7
FP normal control	70.8 ±8.2(ii)	127.8±25.9
exp #2		
FP*[3] #1	10.5 ±0.4(i)	65.8±21.5
FP*[3] #2	10.5 ±0.5(i)	85.0±50.0
FP*[3] #3	11.6 ±0.5(i)	65.6±32.2
FP*[3] #4	9.3 ±0.6(i)	52.2±13.4
FP*[3] #5	9.1 ±0.5(i)	69.0±24.2
FP[4] normal control	74.0 ±6.2(ii)	134.0±25.0
SC[4] normal control	11.2 ±0.6(iii)	147.0±37.5

[1]. Whole blood was washed twice in RPMI-1640 serum free medium. 0.1 ml of 1:3 reconstituted whole blood was used in both experiments to assay for GVH splenomegaly, as described in Materials and Methods.

[2]. 3 FP* birds, 20 weeks of age, made chimaeric with SC red cells at day 12 of embryogenisis after parabiosis

[3]. 5 FP* birds, 7 weeks old, made chimaeric with SC red cells at day 15 of embryogenesis after stem cell injection

[4]. FP and SC control that birds used in these experiments are aged matched birds had been injected at the 15th day of embryogenesis with syngeneic embryonic stem cells.

[5]. 9-12 host embryos were used per experimental group. Groups (i) and (ii) of exp #1 and #2 differ significantly $P<0.001$.
Groups (i) and (iii) of exp #2 are not significantly different $P>0.1$.

in both types of experimental birds. The reaction caused by the injection of lymphocytes from FP* birds into SC embryos is identical with that obtained when SC embryos are injected

with adult SC lymphocytes. The specificity of the reaction is shown by the ability of lymphocytes from chimaeric birds to cause GVH splenomegaly when injected into outbred 12 day old embryos. When lymphocytes from normal and tolerant birds are compared for their ability to cause GVH reactivity on injection into outbred embryos it is found that the tolerant lymphocytes show a lower reactivity. This is most likely to be due to the induction of cross tolerance. This notion is supported by the finding that lymphocytes from tolerant birds are able to cause a very strong reaction in some of the injected embryos whereas in others only a weak one takes place. This variability is reflected in the large standard error of the spleen weight of outbred hosts four days after they are injected with lymphocytes from tolerant donors (see Table 2).

Table 3, shows the results obtained when lymphocytes from 8 SC* birds are tested individually for their ability to cause GVH CAM-POCK reactivity. In this table chimaeras generated at day 12 by parabiosis and day 15 after stem cell injection are included in 2 different experiments. The CAM-POCK assay used in these experiments enumerates single alloreactive lymphocytes that are placed onto the CAM of allogeneic embryos. Lesions or pocks of characteristic morphology and size are formed on the CAM of the embryos within 4 days when the donor immunocompetent lymphocytes differ from the embryos at the MHC region. The CAM-POCK assay is therefore a very sensitive technique in assessing

Table 3. Graft-Versus-Host reactivity of adult chimaeric birds, generated at days 12 and 15 of embryogenesis, as assayed by CAM-POCK assay[1]

Source of donor cells	No of pocks 4 days post inoculation: FP Hosts[4] (mean±SE)		Outbred Hosts[4] (mean±SE)
exp #1			
SC*[2] #1	0 (7)	(i)	12.0± 6.6
SC*[2] #2	0 (8)	(i)	12.6± 4.2
SC*[2] #3	0 (8)	(i)	10.2± 3.4
SC normal control	22.7±6.4	(ii)	31.5± 9.4
exp #2			
SC*[3] #1	0 (8)	(i)	55.0± 9.6
SC*[3] #2	0 (7)	(i)	22.5± 8.7
SC*[3] #3	0 (8)	(i)	24.8±11.4
SC*[3] #4	0 (7)	(i)	24.0± 8.6
SC*[3] #5	0 (8)	(i)	18.7± 6.5
SC normal control	43.5±5.5	(ii)	56.1±15.0

[1]. Whole blood was washed twice in RPMI-1640 serum free medium. 0.1 ml of 1:3 (exp #1) or 1:2 (exp #2) reconstituted whole blood was used in both experiments to assay for GVH CAM-POCK reactivity as described in Materials and Methods.
[2]. 3 SC birds, 16 wks old, made chimaeric with FP red cells at day 12 of embryogenesis after paraboisis.
[3]. 5 SC birds, 3 wks old, made chimaeric with FP red cells at day 15 of embryogenesis after stem cell injection.
[4]. 9-12 host embryos were injected per experimental group. The number of CAMs recovered is given in parenthesis. Groups (i) and (ii) of exp. #1 and #2 differ significantly P <0.001.

the degree and specificity of tolerance. As can be seen in Table 3, complete and specific tolerance was induced in both types of chimaeras as assayed by this GVH reaction.

This specific lack of GVH reactivity holds over a wide range of lymphocyte doses for both types of assay. Although it is known that there are variations from experiment to

experiment as far as the magnitude of GVH reactivity caused by a given donor of normal lymphocytes (Longenecker et al., 1972), it was a regular finding in these studies that CAM-pocks could not be reliably counted if more than 10^6 immunocompetent peripheral blood lymphocytes were inoculated onto the CAM of allogeneic embryos. Table 4, shows two different experiments where the ability of different numbers of lymphocytes to cause GVH reactivity (from 2.5×10^5 to 10^6) from chimaeric and normal birds are compared. It can be seen that in both types of assay the reactivity caused by normal lymphocytes increases when increasing numbers of lymphocytes are inoculated onto allogeneic embryos whereas the reactivity of lymphocytes from chimaeric birds remains constant. A total of 91 SC* and 29 FP* birds were generated and tested individually in different experiments over a period of 4 years.

Complete and specific tolerance was found to be a permanent property of these birds which remained chimaeric over long periods of time. This is demonstrated in Tables 5 and 6 where some of the chimaeric birds generated at day 12 by embryonic parabiosis remain specifically tolerant and chimaeric at 80 and 200 weeks of age. Table 5 also demonstrates an additional property of the chimaeras generated after parabiosis. This is the lack of tolerance to minor histocompatibility antigens. As emphasized under Materials and Methods both types of GVH reactions splenomegaly and the CAM-POCK, measure reactivities caused

Table 4. Graft-Versus-Host reactivity of chimaeric birds as assayed by splenomegaly and chorioallantoic membrane pocks[1]

Source of donor cells	Number of cells	Weight of spleens (in mgs) 4 days post injection: FP Hosts[2] (mean±SE)	Weight of spleens (in mgs) 4 days post injection: Outbred Hosts[2] (mean±SE)
exp #1			
Normal SC[3]	2.5 x 10^5	43.5± 6.08	N.T.
"	5.0 x 10^5	61.2±11.2	N.T.
"	10.0 x 10^5	100.7±18.5	66.0± 6.01
SC*3[3]	2.5 x 10^5	9.1± 0.4	N.T.
"	5.0 x 10^5	9.8± 0.6	N.T.
"	10.0 x 10^5	12.1± 0.8	36.4± 7.9
---	---	11.1± 0.7	

		No of Pocks 4 days post inoculation: FP Hosts[2] (mean±SE)	No of Pocks 4 days post inoculation: Outbred Hosts[2] (mean±SE)
exp #2			
Normal SC[3]	2.5 x 10^5	15.1± 4.8	N.T
"	5.0 x 10^5	31.4± 4.2	N.T
"	10.0 x 10^5	56.6±10.3	35.4±14.5
SC*[3]	2.5 x 10^5	0	10.3± 2.7
"	5.0 x 10^5	0	12.8± 4.8
"	10.0 x 10^5	0	20.1± 3.3
Normal FP	10.0 x 10^5	0	N.T
---	---	0	N.T

[1]. Donor PBLs were purified and assayed for GVH splenomegaly and CAM-POCK reactivity as described in Materials and Methods.

[2]. 9-12 host embryos were injected or inoculated per experimental group.

[3]. Both the 15-day chimaeric and the control bird were 80 weeks old. At this age the experimental bird was 10% chimaeric.

[4]. Both the 15-day chimaeric and the control bird were 48 weeks old. At this age the experimental bird was 10% chimaeric.

N.T. = Not tested.

Table 5. Persistence of specific tolerance to MHC antigens in old chimaeras. Presence of reactivity to minor antigens as measured by GVH CAM-POCK assay in chimaeras generated at day 12 of embryogenesis. Absence of such reactivity in chimaeras generated at day 15 of embryogenesis.

Source of donor cells	% Chimaerism	No of pocks 6 days post injection			
		FP Hosts[2] (mean±SE)		SC Hosts[2] (mean±SE)	
		Large Pocks	Small Pocks	Large Pocks	Small Pocks
exp #1					
FP*[3]	N.T			0	5.1±1.7
SC*[3] #1	30-40	0	6.2±1.8		
SC*[3] #2	30-40	0	3.0±1.6		
exp #2					
FP*[4] #1	10-20			0	5.8±1.3
FP*[4] #2	30-40			0	6.5±1.9
exp #3					
FP*[5] #1				0	0
FP*[5] #2				0	0
FP*[5] #3				0	0
FP normal control				40.3±7.2	N.C.

[1]. Whole blood was washed twice in RPMI-1640 serum free medium. 0.1 ml of 1:3 reconstituted whole blood was used in both experiments to assay for GVH, CAM-POCK reactivity in a 6 day assay.
[2]. 9-12 host embryos used per experimental group. The number of recovered CAMS is given in parenthesis.
[3]. FP* and SC* birds generated after embryonic parabiosis at day 12 of embryogenesis, tested at 200 weeks of age.
[4]. FP* birds generated at day 12 of embryogeneis by parabiosis tested at 20 weeks of age.
[5]. FP* birds generated at day 15 of embryogenesis after stem cell injection, tested at 80 weeks of age.
N.C = Not counted.

by disparities at the MHC B region when assayed 4 days after lymphocyte administration. The appearance of qualitatively different pocks (small without a necrotic center) after the

5th day of lymphocyte inoculation is due to reactivities caused by disparities at minor histocompatibility loci. An interesting observation was made when chimaeras generated after embryonic paraboisis and chimaeras generated after stem cell injection were compared. When lymphocytes from chimaeras generated at day 15 of embryogenesis were assayed for GVH reactivity on the 6th day after lymphocyte inoculation, the absence of small pocks, due to minor histocompatibility differences, was a common finding, see exp #2, Table 5. In contrast, when lymphocytes from chimaeras made by parabiosis were assayed for GVH reactivity 6 days after lymphocyte administration both types of GVH assays revealed reactivity. Table 6 shows the slight increment in spleen weight caused when cells from chimaeric birds are injected into donor type embryos and Table 5 demonstrates the existence of a few small pocks on the CAM of donor type embryos. Since no reactivity against MHC antigens can be seen in older birds by the pock assay (see Table 5) it seems likely that the increase in spleen weight observed at day 6 in the splenomegaly reaction, on injecting cells from older chimaeric birds, is due to reactivity against minor antigens (See Table 6). These differences between chimaeras made after parabiosis and stem cell injection can be easily explained when one takes into account the differences in the ways by which they are produced. When chimaerism is established by stem cell injection about a hundred embryonic donor spleens are

Table 6. Persistence of specific tolerance to MHC antigens in older chimaeras, generated at day 12 of embryogenesis, and the presence of reactivity to minor antigens as measured by splenomegaly[1]

Source of donor cells	Weight of spleens (mean mgs ± SE) 4 days post injection: FP Hosts[2]	Weight of spleens (mean mgs ± SE) 4 days post injection: Outbred Hosts[2]
exp #1		
SC*[3] #1	13.0±0.8(i)	79.0±26.7
SC*[3] #2	10.1±1.2(i)	144.0±49.3
SC*[3] #3	10.1±1.3(i)	75.1±27.8
SC normal control	112.4±3.3(ii)	258.0±28.3

	Weight of Spleens (mean mgs ± SE) 6 days after injection: SC hosts	FP hosts	B^{13}/B^{13} Hosts	B^{1}/B^{13} Hosts
exp #2				
SC*[4] #1		23.6±1.7(i)	83.6±3.2	
SC*[4] #2		32.5±2.1(i)	95.3±3.9	
SC normal control		129.5±5.6(ii)	120.2±5.4	
FP*[5] #1	20.1±1.6(i)		89.8±3.8	120.4±4.8
FP*[5] #2	16.4±1.8(i)		56.1±1.4	81.8±3.7
FP normal control	110.4±4.7(ii)		102.0±4.5	137.0±5.2

1. Whole blood was washed twice in RPMI-1640 serum free mediu medium. 0.1. ml of 1:3 reconstituted whole blood was used in both experiments to assay for GVH spenomegaly in a 4 day assay (exp #1) or in a 6 day assay (exp #2).
2. 9-12 host embryos were used per experimental group.
3. 3 SC* birds, 16 weeks old.
4. The same birds of Table 3, tested a year later, that is at 68 weeks of age.
5. The same birds of Table 2, tested a year later that is at 72 weeks of age. At 20 weeks of age no splenomegaly could be detected in a day 4 assay (see Table 2). Groups (i) and (ii) of exp. #1 and #2, differ significantly P>0.001.

pooled. This experimental protocol leads not only to tolerance to MHC encoded B antigens but also to those coded for by minor histocompatibility loci. In contrast, chimaeras made by parabiosis of two single different embryos leads only to tolerance to MHC B region encoded antigens and to minor histocompatibility antigens that these particular embryos happen to carry.

ii) Ontogeny of GVH immunocompetence

1) Specific absence of GVH alloreactive clones in newly hatched birds injected with allogeneic stem cells at day 15 of embryogenesis

It is clear from the above results that cell-mediated immunity, as assayed by two GVH reactions, is specifically absent in adult stable chimaeras generated at days 12 and 15 of embryogenesis. In addition to this type of analysis the ontogeny of cell-mediated immunocompetence, as measured by the GVH, CAM-POCK assay, was followed in normal birds and in birds injected with allogeneic stem cells at day 15 of embryonic development. This important early phase of immunological reactivity, during which tolerance is induced, is the least studied phase in most similar experimental systems. It was found that as early as day 2 post hatching normal chicken spleen cells contain pock forming cells and their number increases with age. In contrast, spleen cells from young chicks injected as embryos with allogeneic stem cells fail to show any reactivity against the tolerance inducing antigen. Tolerance was shown to be specific as

spleen cells from these birds were reactive against third party embryos (see Fig. 3).

An additional second approach was employed to follow the ontogeny of lymphocytes responding to MHC encoded alloantigens. This involved a combination of an in-vitro and in-vivo assay. Fetal spleen cells (day 18), and spleen cells from 0 day (hatching) and 2 day-old chicks were cultured in-vitro for 48 hours in the presence of the T cell mitogen phytohaemagglutinin in RPMI-1640 serum free medium; their ability to form pocks was subsequently tested by innoculating them onto allogeneic embryos. The data (see Table 7, exp #1) show that fetal spleen cells are not able to form pocks even in the presence of PHA. Spleen cells taken from chicks at the day of hatching, however, show a dramatic increase in their ability to form pocks if PHA is present in the culture medium, in contrast to the absence of reactivity observed if the same cells are not treated with the mitogen. This methodology not only makes possible an earlier identification of pock forming cells (at day 0 rather than at day 2) but also reveals higher numbers of responding cells and thus increases the sensitivity of the assay. Lymphocytes from newly hatched chicks, injected as embryos with allogeneic stem cells, were tested for anti-donor activity by the same procedure. The results (Table 7, exp #2) show that specific alloreactive clones were absent in these chicks even when their lymphocytes were cultured with PHA. Thus, even with this rigorous test, which reveals

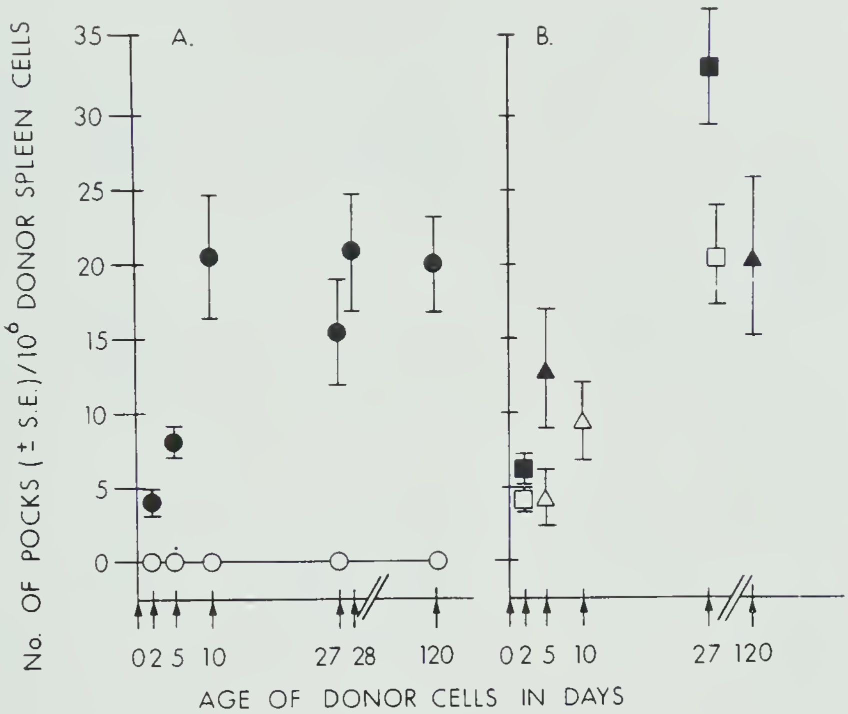

Fig. 3. Ontogeny of GVH reactivity of splenic lymphocytes from normal and chimaeric birds. Donor cells were prepared as described in Materials and Methods. 10^6 spleen cells from donors of different ages were inoculated into various host embryos and the number of pocks formed was counted 4 days later. Lymphocytes from SC birds, injected at day 15 of embryogenesis with FP stem cells, inoculated onto FP embryos: open circles, or onto B^{13}/B^{13} and/or outbred embryos: open squares and triangles respectively. Control lymphocytes from normal SC age-matched embryos inoculated onto FP embryos: closed circles, or onto B^{13}/B^{13} and/or outbred embryos: closed squares and triangles respectively. Vertical bars represent the standard error of the mean. The cell population of the newly hatched chicks is very heterogeneous and sensitive to in-vitro manipulations. The approximate number of white cells per spleen depends on age as follows: day 2; 2.0×10^6, day 3; 4.7×10^6, day 5; 11×10^6, day 10; 27.0×10^6.

Table 7. Use of PHA to detect the low alloreactivity present in the spleen of normal newly hatched chicks. Failure of the mitogen to specifically stimulate alloreactivity in the spleen cells of age matched chicks injected as embryos with allogeneic stem cells.[1]

Source of donor cells	Age of donor cells[2]	PHA	No of Pocks (mean $\pm$ SE) 4 days post inoculation: SC Hosts[3]	FP hosts[3]	B^{13}/B^{13} Hosts[3]	Outbred Hosts[3]
exp #1						
SC normal spleen cells	18	-	0(6)	0(8)		0
"	18	+	0(5)	0(7)		0
"	21	-	0(7)	0(7)		N.T
"	21	+	0(5)	18.5$\pm$3.2		N.T
"	22	-	N.T	0(12)		1.8$\pm$1.6
"	22	+	N.T	16.8$\pm$2.6		19.1$\pm$5.1
exp #2						
SC normal spleen cells	21	-		1.3$\pm$0.8	1.3$\pm$0.3	
"	21	+		14.7$\pm$3.7	13.6$\pm$3.4	
SC* spleen cells	21	-		0(7)	0.8$\pm$0.5	
"	21	+		0.2$\pm$0.2	9.2$\pm$1.2	

[1]. Spleens from 30 normal SC chicks and 30 SC chicks injected with allogeneic FP stem cells were collected at various times as indicated. Single cell suspensions were obtained and cultured either alone or with 10 mg/ml of PHA in RPMI-1640 serum free medium. A number of cells corresponding to 10^6 cells at the beginning of the culture were inoculated onto host embryos as indicated.

[2]. The day of egg incubation is designated as day 0. Hatching occurs on day 21.

[3]. 9-12 hosts were inoculated per experimental group. The figure in parenthesis gives the number of membranes recovered.

alloreactive clones in otherwise non responding lymphoid cell populations, it was not possible to detect anti-donor clones in the spleens of chimaeric chicks on their day of hatching.

2) CAM "Lymphoid" Colonies initiated by spleen cells from very young birds.

The study of the ontogeny of GVH immunocompetence led to the new finding that a fraction of spleen cells from young chicks has the ability to form lymphoid colonies when inoculated onto the CAM of syngeneic or allogeneic embryos. A total of 15 experiments were performed to analyze the ontogeny of pock formation and the ability of spleen cells from young chicks to form lymphoid and haematopoetic colonies. These studies covered the period from the first post hatching day to the third week of life. During this period lymphopoesis could be detected in the spleen. At later times this activity declined. These experiments did not allow a detailed analysis of the phenomenon of lymphopoeisis but they constitute a precise description of the type of activity existing in the spleens of young birds.

Table 8, exp #1, summarises the general findings and describes one experiment where lymphocytes from spleens or peripheral blood were tested for their ability to form pocks and/or "lymphoid" and haematopoetic colonies. Cell suspensions from young spleen cells were prepared by passing the spleens through nylon sieves. These cells tend to

Table 8. CAM-lymphoid colony formation and GVH reactivity of spleen cells from young normal and chimaeric birds.[1]

	No of pocks, small and large white colonies formed 4 days after inoculation								
	SC Hosts (mean±SE) [2]			FP Hosts (mean±SE) [2]			Outbred Hosts (mean±SE) [2]		
Source of Donor cells	LWC	SWC	P	LWC	SWC	P	LWC	SWC	P
A.									
Spleen cells from SC normal birds	-	+	-	+	-	+	+	-	+
	0(7)	5.2±1.0	0(7)	33.8±6.8	0(7)	3.9±1.5	21.8±8.6	0(7)	2.7±1.1
PBLs from SC normal birds	-	-	-	-	-	+	-	-	+
	0(8)	0(8)	0(8)	0(8)	0(8)	14.2±3.0	0(8)	0(8)	4.0±2.0
B.									
Spleen cells from SC* birds	-	+	-	-	+	-	+	+	+
	0(7)	8.5±2.6	0(7)	0(8)	5.9±1.9	0(8)	16.0±1.7	10.3±5.0	1.7±0.7
PBLs from SC* birds	-	-	-	-	-	-	-	-	+
	0(8)	0(8)	0(8)	0(9)	0(9)	0(9)	0(8)	0(8)	5.0±2.1

[1]. Donor spleen cells and PBLs from 5, 2 week old, chicks, either normal or chimaeric, were prepared as described in Materials and Methods. Single spleen cells together with aggregates were inoculated onto various hosts as indicated and the number of pocks (P), small white (SWC), and big white colonies (BWC) formed was counted 4 days later. In the upper line the general pattern of reactivity is described. + indicates presence, - indicates absence. In the lower line the actual numbers from one experiment are presented when 5×10^6 spleen cells and 5×10^5 PBLs from normal and tolerant birds are tested for reactivity.

[2]. 9-12 host embryos were used per experimental group. The number in parenthesis represents the number of membranes recovered.

reaggregate rapidly after their dissociation into single cells. When both single cells and aggregates are inoculated onto the CAM of B-matched embryos, morphologically distinct small white colonies (SWC) are formed. Histological studies revealed that most of these colonies contain a large number of lymphocytes and in some cases lymphocytes and granulocytes. In addition to small white colonies, a few red colonies are formed; these are found most commonly when spleens from one or two day-old chicks are inoculated onto B-matched CAM. When the same splenic population is inoculated onto allogeneic hosts two additional entities are formed. These are, firstly, large white colonies, designated as LWC, and secondly, pocks of characteristic morphology. The LWCs proved on histological examination to consist mainly of lymphocytes, in contrast to pocks which contain mainly mature granulocytes usually surrounding a necrotic center (Longenecker et al., 1970). This "lymphopoietic" activity is a property of the young spleen, since such activity was never detected in the blood of young or adult birds, or in the thymus. There is one point that should be clarified here. Small white colonies are not found when spleen cells are inoculated onto allogeneic embryos. It seems that this may be only an apparent absence as they may be physically obscured by the big white colonies. The nature of the big white colonies seems to have an immunological basis since they are found only when spleen cells are inoculated onto allogeneic embryos. It seems that the big

colonies are formed when immunocompetent lymphocytes, trapped by the colony forming aggregates, stimulate the "lymphoid stem" cells to prolifere with a resulting increase in the size of the small colonies. These notions are supported by two types of experiments. First, spleen cells from young chicks, injected as embryos with allogeneic stem cells, are unable to form big white colonies when inoculated onto the CAM of allogeneic embryos of the donor type, although they are able to form this type of colony when inoculated onto the CAM of third party embryos, see Table 8, exp #2. Secondly, fractionated spleen cells from normal and age matched tolerant birds give different patterns of reactivity when assayed on B-matched or allogeneic hosts of the donor type for their ability to form pocks or small and big white colonies. Spleen cells were dissociated as before and settled for 20 minutes under gravity. Both the supernate, containing single cells, and the aggregate containing fraction were tested for activity. The single cell containing fraction from normal chicks was able to form pocks when inoculated onto allogeneic embryos. This activity was specifically absent in the same fraction of lymphocytes obtained from tolerant birds. On the other hand, when the aggregate-containing fraction was inoculated onto B-matched or allogeneic hosts only small white colonies were formed, indicating that the fractionation procedure separated two cell types required to initiate the big white colonies reported in the previous experiment. Interestingly the same

Table 9. CAM-lymphoid colony formation and GVH reactivity of fractionated spleen cells from young normal and chimaeric birds.[1]

Source of Donor cells	No of Pocks, Small and Big white colonies formed 4 days after inoculation								
	SC Hosts (mean±SE)[2]			FP Hosts (mean±SE)[2]			Outbred Hosts (mean±SE)[2]		
	BWC	SWC	P	BWC	SWC	P	BWC	SWC	P
SC normal (S)[2]	0(8)	0(8)	0(8)	0	0	37.4±5.5	N.T	N.T	N.T
SC normal (A)[3]	0	43.3±6.5	0	0	19.3±4.6	0	0	40.5±10.5	0
SC* (S)[2]	0(8)	0(8)	0(8)	0	0	0	0	0	9.0±2.8
SC* (A)[3]	0	20.6±4.0	0	0	13.8±6.2	0	0	21.7±6.9	0

[1]. 10 day-old, donor spleen cells from normal and chimaeric birds were dissociated into single cell suspension and allowed to settle for 20 min under gravity.

[2]. 5×10^6 spleen cells from the single cell containing fraction (1/10 spleen equivalent) designated as S, were tested for their ability to form Pocks (P) and small (SWC) and big white colonies (LWC).

[3]. 1.2 spleens equivalent of the aggregate-containing fraction, designated as A, were tested also for the above type of activity.

[4]. 9-12 host were used per experimental group. In parenthesis the number of membranes recorded.

aggregate-containing fraction, derived from spleen cells of tolerant birds, was able to cause the formation of small colonies in both B-matched and allogeneic donor type hosts in a manner indistinguishable from cells from the age-matched normal birds. The above results are illustrated in Table 9.

b. Humoral Immunity

I) Specific absence of alloreactive antibodies in chimearas generated at days 12 and 15 of embryogenesis.

Three approaches were used to analyze the state of responsiveness, at the humoral level, of chimaeras, made at days 12 and 15 of embryonic development towards donor MHC antigens. The first was the direct measurement of alloantibody in the serum of otherwise untreated chimaeras from the third week of age to the third year of life. The second was the measurement of allo-antibody in the serum of chimaeras which had been challenged with donor type cells carrying the MHC encoded antigens, under conditions which are known to elicit a good humoral response in normal birds. A third approach was to test for humoral blocking activity in the sera of chimaeras. It was determined whether such sera could specifically block the <u>in-vivo</u> GVH splenomegaly reaction. The latter results will be described in the next section.

None of the sera from chimaeras generated at day 12 of embryogenesis by parabiosis contained any haemagglutinating

antibodies against donor type alloantigens at any stage of their adult life. The majority of the chimaeras generated at day 15 of embryonic development displayed humoral non-reactivity against donor type cells. 22% of this class of chimaeric birds, 8 out of 36, developed specific anti-donor type antibody which could be detected as early as the third week post hatching. The immunological and pathological characteristics of these birds will be described in the next chapter together with the properties of the chimaeras generated at day 17 of embryogenesis. It was found recently that sera from adult normal birds contain natural antibodies against MHC encoded alloantigens of the species, other than those expressed as self antigens. A more sensitive indirect technique is required to reveal these antibodies as direct haemagglutination is never found in these sera (Longenecker et al., 1979). Given this fact, it was thought that, if the chimaeric model represents an experimentally induced condition analogous to that of self tolerance, it should behave similarly in that antibodies should not be found against antigens of the donor type strain. It was therefore considered worthwhile to characterize the sera from chimaeric birds by the direct and indirect technique for the presence or absence of such antibodies.

The results are shown in Table 10, where the sera from a group of 12 birds, all positively typed as chimaeras (see Fig. 2), were tested for direct and indirect haemagglutinating antibodies specific for donor type

Table 10. Serum agglutinin activity of chimaeras generated at day 15 of embryogenesis

Type of sera tested[1]	Direct[2] and (indirect[3]) agglutination titers assayed on different red cells: FP	B^{13}/B^{13}	B^{19}/B^{19}
SC* #1	0(0)	0(1/2+)	0(1/8±)
" #2	0(0)	0(1/1±)	0(1/16±)
" #3	0(1/4+)	0(1/1±)	0(1/4+)
" #4	0(0)	0(1/1+)	0(1/2+)
" #5	0(0)	0(0)	0(1/2+)
" #6	0(0)	0(0)	0(1/1+)
" #7	0(1/2)	0(0)	0(1/2+)
" #8	0(0)	0(0)	0(1/1+)
" #9	0(0)	0(1/1+)	0(1/4+)
" #10	0(0)	0(1/2+)	0(1/2+)
" #11	1/4±(N.T)	N.T(N.T)	N.T(N.T)
" #12	1/4+(N.T)	N.T(N.T)	N.T(N.T)
SC anti FP	1/32(1/1024)	N.T	N.T

[1]. Sera were collected from 6 week old chimaeras.
[2]. 0.1 ml of chimaeric sera were serially diluted two-fold in PBS and 0.1 ml of 0.5% red cells were added in each well as indicated. The plates were incubated for 45 minutes at room temperature and red cell agglutination was directly read through a mirror.
[3]. 100 ul of chimaeric sera were serially diluted two-fold in PBS and 0.1 ml of 0.5% red cells were added to each well. Plates were incubated for 45 minutes at room temperature and then washed twice with 0.1 ml of PBS. Cells were then transferred to new V-bottom plates and 0.1 ml of developing Horse anti chicken serum was added. 45 minutes later red cell agglutination was read through a mirror. The developing serum did not cause any red cell agglutination when tested against the above red cells.
\+ signifies complete agglutination,
± partial agglutination.

alloantigens or third party alloantigens. Although the indirect haemagglutination assay can increase the titer of a SC anti-FP serum from 1:32 to 1:1024 it failed to reveal positive anti-FP reactivity in the majority of SC* chimaeras. 60% of them showed indirect red cell agglutination activity against B^{13}/B^{13} red cells, and all of

them showed a positive reaction when tested against B^{19}/B^{19} red cells. It should be mentioned here that when sera from normal birds are tested for natural alloantibodies higher titers are generally observed. The sera examined in these experiments came from mature 6 month old birds whereas the chimaeric birds were only 6 weeks old. This difference in age might account for the low alloantibody titers observed against third party red cells in the sera of chimaeric birds. Another and equally likely explanation is that cross tolerance exists at the humoral level as was found at the cell-mediated level. Such cross tolerance could be responsible for the lower antibody titers found, against third-party alloantigens, in the chimaeras.

The second approach was to determine whether the chimaeras were specifically unresponsive at the humoral level by deliberately challenging them with adult donor type cells. This experiment was part of a general experimental plan, the results of which will be presented in the next section, (see Table 14, group 4). For the purpose of this section the relevant group will be described. A group of 4 chimaeras, generated at day 15 of embryogenesis and which did not contain any detectable anti-donor strain antibody, served as hosts for challenge with donor type FP adult red blood cells, together with red cells from B^{14}/B^{14} donors. Each host bird was injected at days 0, 2, 4 and 10 with equal amounts of red cells obtained from a total of 8 mls of FP and 8 mls of B^{14}/B^{14} whole blood. The sera obtained at

day 12 after the first injection did not contain any reactivity against donor type FP red cells although these birds did make anti-B^{14}/B^{14} alloantibodies.

C. Lack of specific reactivity in the absence of active suppression.

From the evidence presented above it is clear that the two major classes of immune reactivity, cell-mediated and humoral, are specifically absent in all chimaeric birds generated at day 12 of embryonic development and in the majority, (78%), of the chimaeras generated at day 15 of embryogenesis. The question of whether an active mechanism is responsible for maintaining the unresponsive state was addressed either directly by trying to determine whether there are humoral or cellular elements in the chimaeras that are able to actively inhibit normal immune responses, or indirectly by attempting to break the unresponsive state by various treatments which had been shown in other systems to be effective in breaking tolerance.

i) Absence of various "blocking factors" in the sera of chimaeras.

The inhibition of GVH splenomegaly reactivity is a particularly useful tool in searching for the presence of antibodies directed against lymphocyte surface antigens. Although there are no systematic studies on the class of antibody mediating this inhibition, it seems that this is a property of hyperimmune sera (Fredericksen et al., 1977).

The haemagglutination titer against red cells of the immunizing type is usually accepted as an index of antibody formation against MHC encoded antigens in chickens as these antigens are expressed in high concentrations on red cells in this species. The equivalent titers for alloantigens present on lymphocytes are not known. The class of antibody able to inhibit splenomegaly and the concentration of the relevant MHC encoded B alloantigens on lymphocytes are not known and this should be kept in mind when the results of these types of experiments are evaluated. A second point to be made here concerns the mechanism of action of inhibition. It seems that antibody directed against cell surface antigens of responder lymphocytes prevents them from homing into the spleen of the host embryos and thereby prevents the splenomegally reaction from taking place (Lydyard and Ivanyi, 1974, and Pink and Migianno, 1977).

The possible role of putative factors present in the serum of chimaeras in maintaining the unresponsive state was investigated at two levels. First, it was determined whether chimaeric serum could inhibit the reactivity of normal host type lymphocytes, and secondly whether such serum could inhibit the reactivity of normal donor type lymphocytes. Numerous experiments were performed in which the inhibitory ability of sera from different tolerant chimaeric birds were tested individually at different stages of adulthood starting from the fourth week post hatching to the third year of age. All such attempts were negative. In Table 11

Table 11. Absence of blocking activity in the serum of chimaeric birds[1]

Source of 10^6 donor lymphocytes	Type of serum	Agglutination titer of serum tested on red cells: FP	SC	Weight of spleen (mgs) 4 days after injection (mean$\pm$SE) of: FP hosts[4]	B^{13}/B^{13} Hosts[4]
SC normal	SC normal	0	0	110 $\pm$10.7	
"	SC anti FP [2]	1/16	0	122 $\pm$18.4	
"	SC* #1 [3]	0	0	137 $\pm$20.2	
"	SC* #2 [3]	0	0	157.1$\pm$21.7	
"	SC* #3 [3]	0	0	173.0$\pm$29.7	
"	SC* #4 [3]	0	0	142.5$\pm$14.8	
FP normal	SC normal				76.8$\pm$ 6.2
"	SC anti FP				14.8$\pm$ 1.1
"	SC* #1				86.3$\pm$ 9.6
"	SC* #2				77.0$\pm$11.7
"	SC* #3				89.5$\pm$ 5.1
"	SC* #4				104.6$\pm$ 9.2

1. 10^6 purified peripheral blood lymphocytes were incubated in 1 ml of 50% serum obtained from various donors as indicated, for one hour at room temperature. After washing once these cells were injected into different hosts as indicated, and their cellular activity was measured four days later by weighing the excised spleens of the appropriate recipients.
2. The SC anti-FP serum tested was obtained after hyperimmunization of SC birds with FP whole blood and subsequently absorbed with SC type cells.
3. Sera from 8 week-old chimaeric birds.

the behavior of 4 such sera is shown together with the positive inhibition obtained when normal lymphocytes were treated with a low titer, 1:8 of hyperimmune serum. Although there are no systematic studies on the class of antibody inhibiting the GVH reaction, as mentioned before, it was observed through the course of these studies that sera from birds immunized under the standard protocol with allogeneic whole blood do not inhibit the splenomegaly reaction for a period of 10 to 30 days after the first injection, although these sera have positive red cell agglutination titers after the 10th day. The biological role of serum alloantibodies will be further discussed in the next chapter in which it will be made clear that where the majority of chimaeras produced at day 17 of embryonic development display positive haemagglutination titers against donor type red cells.

ii) Absence of detectable suppressor activity in chimaeric lymphocytes.

The possible role of inhibitory cells in maintaining the tolerant state in the chimaeras was investigated in a series of experiments where lymphocytes from tolerant birds were tested for their ability to suppress the GVH reactivity of normal lymphocytes. Inhibitory cells were never found in the peripheral blood lymphocytes of chimaeras generated either at day 12 or day 15 of embryogenesis, although many experiments were performed where different cell concentrations were examined.

Table 12. Absence of cell-mediated suppression in chimaeric birds.[1]

	Number of cells inoculated		No of Pocks 4 days post inoculation (mean±SE)	
	Normal SC[2]	SC*[2]	FP Hosts[3]	Outbred Hosts[3]
exp #1	2.5×10^5	---	16.8± 5.2	
	5.0×10^5	---	24.8± 5.1	
	10×10^5	---	46.1± 9.9	11.3± 3.0
	---	5.0×10^5	0	
	---	10×10^5	0	
	---	20×10^5	0	8.0± 1.6
	2.5×10^5	5.0×10^5	10.4± 2.6	
	2.5×10^5	10×10^5	10.3± 2.3	
	5.0×10^5	10×10^5	24.2± 8.2	
	5.0×10^5	20×10^5	30.1± 7.5	
exp #2	2.5×10^5	---	15.8± 4.85	
	5.0×10^5	---	31.4± 4.2	
	10×10^5	---	56.6±10.3	35.4±14.5
	---	2.5×10^5	0	
	---	5.0×10^5	0	
	---	10×10^5	0	20.1± 3.3
	2.5×10^5	2.5×10^5	17.1± 2.7	
	2.5×10^5	5.0×10^5	12.3± 1.4	
	5.0×10^5	5.0×10^5	26.5± 3.6	
	5.0×10^5	10×10^5	25.6± 3.0	
exp #3	2.5×10^5	---	14.3± 2.2	
	5.0×10^5	---	31.4± 7.8	
	10×10^5	---	40.8± 5.7	32.8± 8.6
	---	10×10^6	0	22.1± 3.6
	5.0×10^5	5×10^5	34.3± 5.5	
	5.0×10^5	10×10^5	35.3± 6.8	
	5.0×10^5	20×10^5	33.1± 3.4	

[1]. Peripheral blood lymphocytes were tested as indicated for their ability to inhibit the pock forming ability of normal peripheral blood lymphocytes.
[2]. The chimaeric birds and the control birds used were 4 months old. All three chimaeras were made at day 15 of embryogenesis and they had no detectable anti-FP antibodies.
[3]. 9-12 host embryos were inoculated per experimental group.

Table 12 presents the results obtained when lymphocytes from 3 different chimaeric birds were tested individually for their ability to inhibit the pock forming ability of

normal lymphocytes. As can be seen in these experiments a striking "neutrality" of tolerant lymphocytes was observed when they were mixed with normal lymphocytes. A total of 19 chimaeric birds were individually tested in a similar manner employing either the splenomegaly or the CAM-POCK assay. The results were identical with the ones presented above.

iii) Stability of unresponsiveness after various treatments of chimaeric cells.

From the results presented in the previous two sections it is clear that attempts to detect either humoral or cellular inhibitory activity in these unresponsive chimaeras were unsuccessful. Although efforts to detect suppressor activity were extensive the immunological unresponsiveness of these birds could in principle be still explained by a low but undetectable level of inhibition. A second indirect approach was employed in the search for inhibitory activity. It was argued that should unresponsiveness be due to inhibition rather then deletion some treatments might reverse tolerance and reveal immunological reactivity. A demonstration of depressed clones would imply that a continuous active mechanism maintains the tolerant state and be inconsistent with deletion. Several different approaches have been used in these experiments. They have been based mainly on observations from other systems where unresponsive lymphocytes show reactivity after various treatments. These systems were extensively discussed in the introduction. In brief, humoral unresponsiveness to SRBCs induced neonatally

in rats, can be reversed if lymphocytes from adult unresponsive animals are preincubated for 12 hours in-vitro before being transferred to irradiated syngeneic hosts (McGregor et al., 1967). Humoral unresponsiveness to polymerised flagellin of Salmonella adelaida, induced in-vitro, is reversed when the antigen or antigen-antibody complexes are removed from the surface of lymphocytes by trypsin treatment (Diener and Feldman, 1972). Finally, it was reported recently that T cell polyclonal activators could induce in-vitro killer cells specific for donor strain alloantigens when incubated with lymphocytes from adult mice in which tolerance to H-2 antigens was induced neonatally (Waterfield et al., 1978). This last approach seems particularly useful as work presented here has shown that PHA can dramatically increase the ability of lymphocytes from newly hatched chicks to display GVH reactions.

Table 13 summarizes experiments performed in efforts to reverse tolerance. Lymphocytes from an adult chimaera were: i) incubated for 48 hours in-vitro and then assayed for GVH splenomegaly reactivity, ii) treated with trypsin, washed extensively and incubated for 48 hours in vitro, and then assayed for GVH reactivity and iii) cultured for 48 hours in the presence of phytohaemagglutinin and then assayed for GVH reactivity. It can be seen that none of these treatments was successful in revealing any activity with specificity against the tolerated strain. A total of 8 chimearic birds, with ages ranging from 20 to 40 weeks old, were tested

Table 13. Stability of unresponsiveness after various treatments of chimaeric cells.[1]

Source of Donor Cells	treatment with: Trypsin[2]	PHA[3]	Weight (in mgs $\pm$ SE) of spleens 6 days after injection[4]: FP Hosts	Outbred Hosts
SC*	-	-	26.1$\pm$ 2.4	70.1$\pm$29.0
"	+	-	24.0$\pm$ 1.8	88.2$\pm$38.6
"	+	+	27.4$\pm$ 4.3	65.8$\pm$24.0
SC normal control	-	-	188.0$\pm$12.4	130.0$\pm$21.0
	+	-	169.0$\pm$19.2	N.T
	+	+	158.0$\pm$16.2	N.T
FP normal control	-	-	26.2$\pm$ 3.8	N.T
	-	+	42.0$\pm$ 9.0	N.T

[1]. Cultured PBLs were injected into host embryos as indicated. Purified PBLs were cultured for 2 days in microtiter plates at 1.5 X 10^7 cells/ml in RPM-1640 serum free medium. Some cells were treated in additional ways as described in 2 and 3. The number of cells injected into each host embryo corresponds to 10^6 cells at the beginning of the culture.
[2]. Purified PBLs were treated with 0.25% trypsin at 37°C at a concentration of 1.5 x 10^7 cells/ml. After two washes they were cultured as described in 1.
[3]. Cells trypsinized as described in 2 were cultured under the same conditions as in 1 except that 20 mg/ml of PHA was added to the medium.
[4]. 9-12 host embryos were injected per experimental group.

individually in a similar manner with identical results.

iiii) Adult chimaeric birds succumb to GVH reactivity on challenge with donor type lymphocytes to which they are tolerant.

In addition to the above described experiments, which involved efforts to reveal activity by incubating lymphocytes from tolerant birds in various ways, additional

experiments were made at the level of the whole bird. It has been shown that unresponsiveness to SRBCs at the humoral level, induced neonatally in rats, can be reversed by giving allogeneic lymphocytes to the experimental animals. The antibody producing cells are of host origin (McCullagh, 1972). An effort to break tolerance in an analogous way was attempted and the results are shown in Table 14.

Chimaeric birds were challenged as adults by i.v. injection of a relatively few lymphocytes of the donor type strain in the form of whole blood using a protocol similar to that employed to immunize birds for the production of alloantibodies. All chimaeras so injected in that manner succumb to a lethal GVH reaction 3 weeks after the first injection (group 2) in contrast to normal birds. One bird of this group, bled a day before its death, was typed for erythrocyte chimaerism. The typing revealed that the only surviving red cells at that stage were of the donor type. In contrast, when chimaeras were challenged with a mixture of purified red cells of donor type together with red cells of a third party strain B^{14}/B^{14} they remained healthy for a period of 3 months until they were terminated. These birds were unable to form antibodies against the injected donor type red cells, although they formed antibodies against the red cells of the B^{14}/B^{14} strain (see group 4). Group 3 is a control demonstrating that B^{14}/B^{14} third party whole blood is not the cause of GVH death observed in group 2. All members of this group remained healthy until 3 months of age

Table 14. Adult chimaeric birds succumb to GVH reactivity on challenge with donor type lymphocytes to which they are tolerant.

			Direct agglutination titers on red cells of:		
Group No	Source of Serum	Immunization with:	FP	B^{14}/B^{14}	Death due to GVH
	SC normal			N.T	
	#1		1/128±	N.T	-
	#2	FP whole	1/16+		
G.1	#3	blood	1/64+	N.T	-
	#4		1/32+	N.T	-
	#5		1/32±	N.T	-
	SC*				
	#1		0	1/8+	+
	#2	FP and	0	1/16+	+
	#3	B^{14}/B^{14}	0	1/16±	+
G.2	#4	whole blood	1/1±	1/64±	+
	#5		0	1/8	+
	#6		0	1/16	+
	#7		0	1/8	+
	#8		0	1/32	+
	SC*				
	#1		0	1/16+	-
	#2	B^{14}/B^{14}	0	1/8±	-
G.3	#3	whole blood	0	1/32+	-
	#4		0	1/16±	-
	SC*				
	#1		0	1/32+	-
	#2	FP and	0	1/16±	-
G.4	#3	B^{14}/B^{14}	0	1/16±	-
	#4	red cells	0	1/6+	-

G.1. Adult 12 week old normal SC birds were challenged with a total of 8 mls of whole blood from age matched FP birds, as described in Materials and Methods. Red cell agglutination was measured 10-12 days after the first injection.
G.2, 3 and 4. 12 weeks old SC* birds were challenged with different types of donor cells as indicated, in a similar manner to group 1. In these groups, chimaerism ranged from 10-40%. (see Fig. 2). None of these birds had any detectable anti FP antibodies prior to immunization.

when they were terminated. The above experiments demonstrate a lack of resistance in the chimaeric birds against the

deleterious consequences of the immunocompetent donor type lymphocytes and argue against a form of unresponsiveness that can be reversed by a chronic allogeneic stimulus.

D. The importance of antigen persistence for maintaining the unresponsive state.

The importance of antigen persistence for maintaining the unresponsive state was demonstrated in this system. Tolerance was induced in a group of 8 birds by injecting embryos at day 15 of embryogenesis with allogeneic "adherent" type embryonic spleen cells. These cells were obtained from the day 15 embryonic spleen after fractionation into "adherent" and "non adherent" types on petri dishes for 2 hours at 37°C. Such "adherent" cells are able to initiate erythropoetic colony formation on the CAM of chicken embryos (Keller et al., 1979). Upon intravenous injection to allogeneic embryos they fail to induce any detectable red cell chimaerism. This is illustrated in Fig. 4 where the counts obtained with red cells from SC birds injected with FP adherent type cells are in the same range of counts as those obtained when SC normal red cells are treated with anti-FP serum.

These birds, which had been injected as 15-day old embryos with allogeneic "adherent" type embryonic spleen cells, were specifically tolerant against the FP MHC encoded alloantigens when tested at 2 weeks of age. Nevertheless, 5 out of 6 of these birds could react against donor MHC

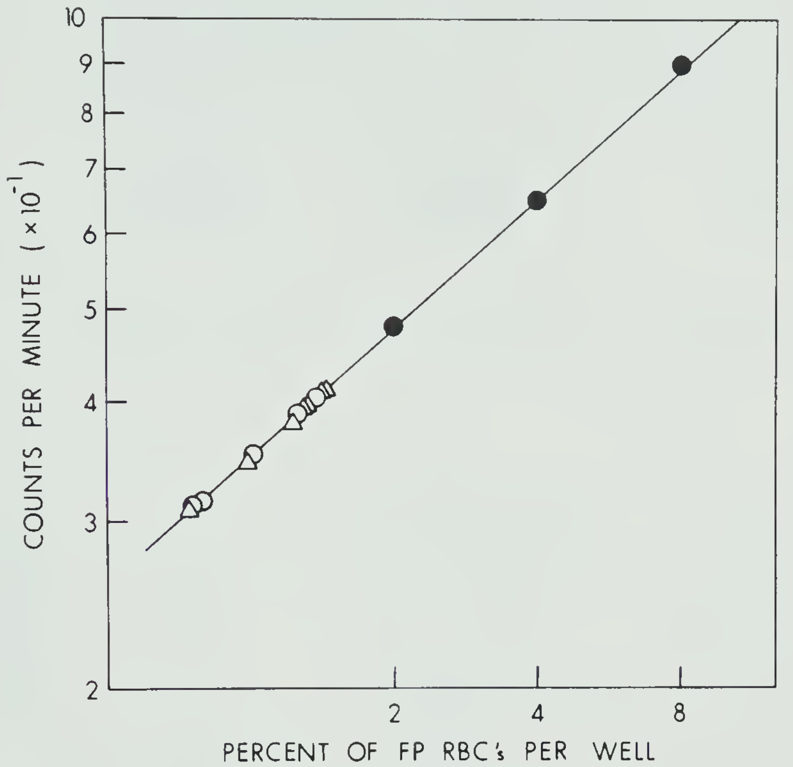

Fig. 4. Determination of chimaerism in a group of birds that had been injected as embryos with allogeneic "adherent" type embryonic spleen cells.
Closed circles represent the standard curve generated by reacting artificial mixes of SC and FP red cells with anti-FP serum. A total of 10^7 red cells were used per well. For details of the assay, see Materials and Methods. Open circles represent the number of counts from 5 control SC birds following incubation of 10^7 red cells with anti-FP serum. Open triangles represent the number of counts from 7, 4 week old SC birds injected as embryos with allogeneic "adherent" type embryonic spleen cells.

antigens when tested at 16 weeks of age (see Table 15), indicating the spontaneous breakage of tolerance.

Table 15. GVH CAM-POCK reactivity of lymphocytes from birds injected with "Adherent" type embryonic spleen cells at day 15 of embryogenesis[1]

	No of Pocks formed (mean ± SE) after inoculation of lymphocytes from:			
	2 week-old chick on:[3]		16 week-old birds on:[3]	
Source of donor cells	FP Hosts[4]	Outbred Hosts[4]	FP Hosts[4]	B^{14}/B^{14} Hosts[4]
SC normal control	36.5± 4.4	56.1±15.0	43.1± 4.6	N.T
SC injected with FP "adherent" type cells #1	0	55.0± 3.6	3.7± 1.2	N.T
#2	0	22.0± 8.7	9.6± 1.7	N.T
#3	0	N.T	8.3± 1.7	N.T
#4	0	N.T	14.0± 3.5	N.T
#5	0	24.8± 1.4	17.8± 3.6	N.T
#6	0	18.7± 6.5	0.3± 0.3	10.0± 1.5

[1]. 1.2 x 10^7 FP "Adherent" type embryonic spleen cells, prepared as described under Materials and Methods, were injected into 15 day old embryos. All members of this group had undetectable chimaerism (less than 1%) as estimated by radioimmunoassay.
[2]. 0.1 ml of 1:3 dilution of whole blood was inoculated onto host type embryos as indicated.
[3]. 0.1 ml of 1:5 dilution of whole blood was inoculated per experimental group.

E. Summary

In this section a system was described in which long term erythroid chimaeras were generated at two different stages of embryonic development. All chimaeras produced at day 12 embryogenesis by parabiosis, and the majority of the

chimaeras (78%) produced at day 15 by stem cell injection, displayed specific tolerance to donor MHC encoded alloantigens at both the cell-mediated and humoral level. Specific tolerance was detectable at the time of hatching which is the time that alloreactivity can be first detected in the spleens of normal chicks. Healthy experimental birds remained chimaeric and specifically tolerant for very long periods of time. The importance of antigen persistence for maintaining the tolerant state was shown in a group of birds in which detectable red cell chimaerism was not produced. In this case tolerance was spontaneously lost after a certain period of time. The specific cell-mediated and humoral tolerance displayed by these birds was shown to be independent of detectable active humoral and cellular suppressor mechanisms. Attempts to reverse the tolerant state by various in-vitro treatments and by the in-vivo production of an allogeneic effect were unsuccessful. These observations further support the previous direct evidence for an absence of suppressor mechanisms actively maintaining the tolerant state. A different class of observation was made during the ontogenic studies of GVH reactivity. This is the finding that spleen cells, taken from birds in their first 3 weeks of age, were able to initiate lymphoid-like colonies on the CAM of syngeneic or allogeneic embryos.

2. <u>Chimaeras generated at day 17 of embryogenesis</u>

A. Establishment and estimation of chimaerism

Chimaeras were generated at day 17 of embryogenesis by

intravenous administration of 17 day-old allogeneic haematopoetic stem cells of spleen origin. The donor cells were prepared in the same manner as the stem cells used to produce chimaeras made at day 15 of embryogenesis. Successful chimaerism, ranging from 5 - 30%, was induced at this developmental stage. The degree of chimaerism was estimated by both the red cell agglutination and cellular radioimmunoassays. A total of 22 SC birds chimaeric with FP red cells were produced in this way, 9 and 13 birds being produced on two separate occasions.

B. Immunological characteristics

a. Young chimaeras display specific unresponsiveness for GVH reactivity in the presence of anti-donor strain humoral allo-antibodies.

The lymphocytes from all chimaeras, produced at day 17 of embryogenesis, display complete and specific unresponsiveness when tested for GVH reactivity as young adults. In contrast with the chimaeras generated at earlier stages of embryogenesis, however, they produce alloantibodies which are detectable after three weeks of age. Table 16 shows the immunological properties of 9 chimaeric birds tested at the 7th week of age. Successful chimaerism was induced in all members of this group and ranged from 10 - 30%. Lymphocytes from 5 randomly chosen birds, from this group, showed complete and specific unresponsiveness when tested for pock formation in donor type embryos. Identical results were obtained when

Table 16. Immunological properties of chimaeras generated at day 17 of embryogenesis.[1]

Source of donor lymphocytes cytes and sera	% chimaerism[2]	CAM-pocks (mean ± SE) enumerated 4 days after lymphocyte inoculation, on: FP hosts[3]	SC hosts[3]	Outbred[3] hosts	Direct agglutination titers measured on red cells of: FP (B^{15}/B^{21})	SC (B^2/B^2)
SC normal control[4]	0	51.3±6.9	0(5)	18.2± 7.8	0	0
SC* #1	10-20	0(8)	0(5)	12.3± 4.6	0	0
" #2	10-20	0(7)	0(6)	9.5± 3.5	0	0
" #3	20	0(8)	0(6)	22.4± 4.9	1/2+	0
" #4	10-20	0(8)	N.T.	16.6± 4.6	1/16±	0
" #5	10-20	0(6)	N.T.	27.4±15.6	0	0
" #6	30	N.T.	N.T.	N.T.	0	0
" #7	10	N.T.	N.T.	N.T.	1/16±	0
" #8	20	N.T.	N.T.	N.T.	1/2+	0
" #9	30	N.T.	N.T.	N.T.	1/16+	0

[1]. Lymphocytes from 7 week-old SC birds, made chimaeric at day 17 of embryogenesis, were tested for their ability to cause GVH, CAM-pock reactions upon inoculation onto appropriate host embryos, as indicated. The sera from the same birds were tedted for their ability to agglutinate red cells of the donor or host type strain.

[2]. Chimaerism in this group of experimental birds was estimated by red cell agglutination as described in Materials and Methods and in Table 1.

[3]. 9-12 host embryos were used per experimental group. The number in parenthesis represents the number of recovered membranes.

[4]. Direct red cell agglutination titers were never found in numerous control birds.

N.T. = not tested

lymphocytes from 6 randomly chosen birds of the second group of 13 birds were tested in a similar manner. As can be seen in Table 16, about 65% of the birds in this category display humoral immunity specific for alloantigens of the donor type strain. The red cell agglutination titers of these birds were low, ranging from 1/2 to 1/32 and were sensitive to 2ME treatment. They were therefore probably due to IgM antibody.

b. Characterization of serum antibodies.

An attempt was made to characterize the specificity of the antibodies formed in young chimaeras. It was essential to find out if the anti-donor strain antibodies, presented in Table 16, represent antibodies directed against antigens encoded by the MHC region. Sera from different chimaeric birds, with agglutination titers against donor type red cells, were tested in parallel with specific alloantiserum against a panel of red cells from different birds known to be homozygous for the MHC B region. Two different assays were employed, namely red cell agglutination and the cellular radioimmunoassay. The results are presented in Table 17 and Fig. 5. Both assays gave comparable results. The antibodies found in the chimaeras seem to be directed against the B region encoded alloantigens. This conclusion is strengthened by the finding that all sera from chimaeric birds gave a very weak reaction against line N red cells. This line carries the B^{21} allele which strongly cross-reacts with the B^{2} allele. In general, all sera tested gave a pattern of reactivity which is characteristic of antisera

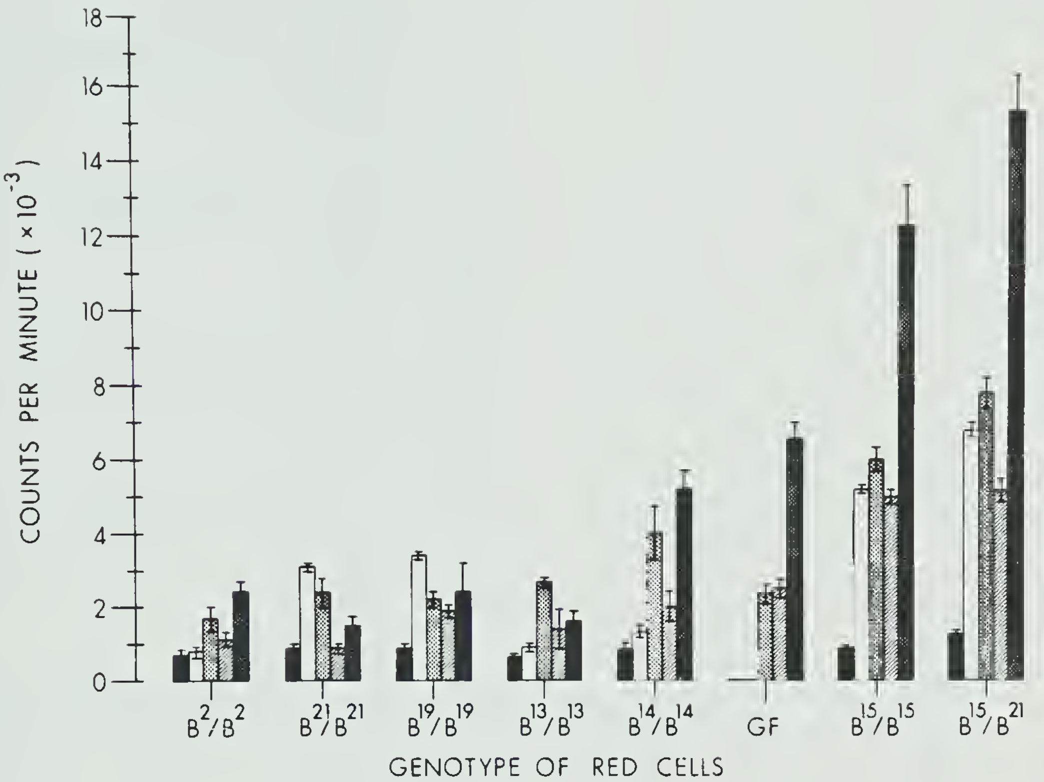

Fig. 5. Specificity of serum antibodies from chimaeric birds tested by cellular radioimmunoassay. Sera from 3 different SC* birds 8 - 10 weeks old, generated at day 17 of embryogenesis, were tested for their ability to bind to red cells of different genotypes. 0.1 ml of undiluted sera from 3 different chimaeric birds and one SC control bird as well as 0.1 ml of 1:20 SC anti FP serum, were incubated for 45 minutes at R.T. with 10^7 red cells of different genotypes. The cells were washed twice and further incubated with 0.1 ml of ^{125}I-rabbit anti chicken immunoglobulin for 30 minutes at R.T. After a further wash the cells were harvested and counted in a Beckman gamma counter. Closed bars: control SC serum, open bars: SC anti FP serum, dotted, lined and shaded bars: sera from SC* #1, 2 and 3 respectively.

Table 17. Specificity of serum antibodies form chimaeric birds.[1]

Source of donor sera	SC[2] B^2/B^2	Line N[2] B^{21}/B^{21}	B^{19}/B^{192}	B^{13}/B^{132}	B^{14}/B^{142}	GF[2]	B^{15}/B^{152}	FP[2] B^{15}/B^{21}	FP[2] B^{15}/B^{21}
SC control bird	0	0	0	0	0	0	0	0	0
SC anti FP	0	1/2+	1/1+	0	0	N.T.[+]	1.8±	1/8+	1/8+
SC* #1	0	0	0	0	0	1/1±	1/4±	1/4+	1/4+
SC* #2	0	0	0	0	0	1/2±	1/4±	1/8+	1/8+
SC* #3	0	1/4±	0	0	0	0	1/8±	1/4+	1/4±
SC* #4	0	1/1±	0	0	0	1/2±	1/4±	1/4±	1/4±
SC* #5	0	0	0	0	0	1/2±	1/16±	1/8+	1/8+
SC* #6	0	0	0	0	0	1/2	1/8+	1/16±	1/16±

[1]. Sera from 6 different chimaeric birds 8-10 weeks old, were tested against red cells of different genotypes.

[2]. The red cells used were obtained from birds of different lines as described in Materials and Methods, except for the breed GF which is a highly inbred line homozygous for a B allele which strongly cross-reacts with B^{15}.

[3]. Sera #1, 2, 3 and 4 were obtained from chimaeric birds, generated at day 17 of embryogenesis. Sera #5 and 6 were obtained from chimaeric birds generated at day 15 of embryogenesis.

\+ = complete red cell agglutination

± = partial red cell agglutination

N.T. = Not tested.

produced against B locus antigens.

The existence of anti-donor strain antibodies in the sera of these chimaeric birds raises the question of whether the lack of anti-donor GVH reactivity shown by the lymphocytes from these chimaeras, is due to these antibodies. The sera from these birds were tested for inhibitory activity in two ways. First the sera were tested for their ability to inhibit the reactivity of host type lymphocytes. Such inhibition could suggest the presence of anti-idiotypic antibodies able to block host receptors from recognizing donor antigens. Secondly, the sera were tested for their capacity to inhibit GVH reactivity of donor type lymphocytes.

Inhibition of GVH splenomegaly was used as an assay system. The results are shown in Table 18. None of the sera tested was able to inhibit the GVH splenomegaly reaction caused by host or donor type normal lymphocytes although such sera had detectable haemaglutination titers against donor type red cells. It is interesting to note that hyperimmune SC anti FP serum with a red cell agglutination titer of 1/16, was able to inhibit the splenomegaly reactivity of FP type lymphocytes, although chimaeric sera with equivalent or higher titers were unable to do so. This observation suggests that the specificity and/or class of antibody predominantly present in the chimaeric and immune sera are different, the chimaeric sera being ineffective in

Table 18. Absence of blocking activity in the serum of chimaeric birds which have antibodies against red cells of the donor type strain.[1]

Source of 10^6 donor lymphocytes	Type of serum	Agglutination titer of serum tested on red cells of: FP (B^{15}/B^{21})	SC (B^2/B^2)	Weight of spleens (mgs) 4 days after injection (mean $\pm$ SE): FP hosts	B13/B13 B^{13}/B^{13}
SC normal	SC normal	0	0	115.0 ± 13.6	
"	SC anti FP[2]	1/16+	0	119.0 ± 24.0	
"	SC* #1[3]	1/16$\pm$	0	126.0 ± 13.5	
"	" #2[3]	1/4$\pm$	0	127.0 ± 15.7	
"	" #3[3]	1/32$\pm$	0	112.1 ± 17.9	
"	" #4[3]	1/4+	0	105.7 ± 16.3	
"	" #5[3]	1/16+	0	120.2 ± 6.7	
FP normal	SC normal				74.3 ± 9.5
"	SC anti FP				17.5 ± 2.1
"	SC* #1[3]				69.0 ± 5.2
"	" #2[3]				83.5 ± 6.1
"	" #3[3]				77.5 ± 6.7

[1]. 10^6 purified peripheral blood lymphocytes were incubated for one hour at R.T. in 1 ml of 50% serum obtained from various donots as indicated. After washing once, these cells were injected into appropriate hosts and their GVH reactivity measured four days later by weighing the excised spleens of the hosts.

[2]. The SC anti-FP serum tested was obtained after hyperimmunization of SC birds with FP whole blood and subsequently absorbing the serum with SC cells.

[3]. Sera from 8 - 10 week-old chimaeric birds.

causing splenomegaly inhibition.

A very intriguing observation, made in five different experiments, was that peripheral blood lymphocytes, from chimaeric birds that were producing anti-donor strain antibody, are able to inhibit the CAM-pock reactivity of normal host type lymphocytes against donor antigens as shown in Table 19. Unfortunately, the specificity of the inhibition was not ascertained and the observations are not in this sense complete.

c. Pathological characteristics of adult chimaeras. Development of autoimmunity and lymphomas.

83% of the chimaeric birds generated at day 17 of embryogenesis developed severe antibody mediated haemolytic anaemia as they aged. An early sign of the disease was the loss of colour of their normally red comb. Diseased birds have their red cells and lymphocytes coated to different degrees by antibodies depending on the stage of the disease. This is demostrated with a direct Coomb's test as shown in Table 20.A. Sera from these birds contain antibodies which are directed against both host and donor type antigens, demonstrating an autoimmune state, as shown in Table 20.B. It should be mentioned here that, although a high degree of chimaerism is demonstrable in young chimaeras with haemagglutinating antibodies, their red cells are not coated by antibodies as determined by a direct Coomb's test. The disease is long lasting, resulting in the death of the

Table 19. Evidence for suppression of GVH, CAM-pock reactivity by cells from from chimaeric birds containing anti-donor strain antibody.[1]

Source and number of cells inoculated			Direct Serum agglutination titers, measured on red cells of donor type	No of Pocks post inoculation (mean+SE)
Normal SC	SC*[2]	SC*[3]		FP Hosts[5]
exp #1				
5×10^5	-	-	0	31.4$\pm$ 4.2(i)
-	10×10^5	-	0	0
-	-	10×10^5	1/16+	0
5×10^5	10×10^5	-		25.7$\pm$ 3.0(ii)
5×10^5	-	10×10^5		14.3$\pm$ 2.6(iii)
exp #2				
5×10^5	-	-	0	31.1$\pm$ 4.5(i)
-	10×10^5	-	0	0
-	-	10×10^5	1/16$\pm$	0
5×10^5	10×10^5	-		35.3$\pm$ 6.8(ii)
5×10^5	-	10×10^5		18.7$\pm$ 1.9(iii)
exp #3				
Normal FP	FP*[2]	FP*[4]		SC Hosts
5×10^5	-	-	0	40.3$\pm$ 7.2(i)
-	10×10^5	-	0	0
-	-	10×10^5	1/8	0
5×10^5	10×10^5	-		41.2$\pm$12.8(ii)
5×10^5	-	10×10^5		20.2$\pm$ 7.9(iii)

[1]. Peripheral blood lymphocytes were tested as indicated for their ability to inhibit the GVH, CAM-pock forming lymphocytes.

[2]. Chimaeric birds of exp #1, 2 and 3, were generated at day 15 of embryogenesis and they had no detectable anti-FP or anti-SC antibodies.

[3]. Chimaeric birds of exp #1 and 2, were generated at day 17 of embryogenesis and had anti-FP antibodies.

[4]. Chimaeric bird of exp #3 was generated at day 15 of embryogenesis and hard anti-SC antibodies.

[5]. 9-12 host embryos were inoculated per experimental group

exp #1, #2 and #3. Groups (i) and (iii) differ significantly $P<0.05$, $P<0.02$, $P<0.005$ respectively.

Table 20. Nature of antibodies present in chimaeras generated at day 17 of embryogenesis

A. Presence of antibodies on red cells and lymphocytes from sick birds

Direct COOMB'S test[1] (mean counts ± SD)

Source of cells	Red cells	Lymphocytes
SC control[2]	243±86 (i)	1043±54 (a)
SC* #1[3]	638±20 (iii)	1784±140 (b)
" #2[3]	564±76 (ii)	1877±257 (b)
" #3[3]	405±18 (ii)	2397±260 (b)
" #4[4]	2201±106 (iii)	6709

B. Presence of antibodies in sera from sick birds directed against both donor and host type antigens.

Indirect COOMB'S test[5] (mean counts ± SD)

Source of donor serum	Lymphocytes of: SC(B^2/B^2)	FP(B^{15}/B^{21})	B^{21}/B^{21}	B^{13}/B^{13}
SC control	367±17	1073±49	436±26	715±122
SC anti FP[6]	455±16	2002±296	729±52	729±73
SC* #1[7]	2587±366	9075±160	5802±1392	3508±376
" #2[7]	2548±260	6448±576	4306±488	3736±282
" #3[7]	3158±348	7768±1056	4075±847	4095±519

1. 10^7 separated red and white cells form peripheral blood were washed twice with PBS and incubated with rabbit anti-chicken immunoglobulin at R.T. for 45 minutes. The washing procedure was repeated and the cells were harvested and counted on a Beckman gamma counter.
2. The mean counts obtained when red cells or lymphocytes from 2 control birds were incubated with rabbit anti chicken immunoglobulin.
3. Cells collected from three adult birds which had macroscopic signs of anaemia.
4. Cells from a very ill bird, collected two weeks before its death.
5. 10^7 separated peripheral blood lymphocytes from birds of different genotypes were incubated for 45 minutes with 0.1 ml of test sera as indicated. The cells were washed twice and further incubated with 0.1 ml of rabbit anti chicken immunoglobulin. After rewashing the cells were harvested and counted as before.
6. 1:100 dilution of SC anti FP serum, absorbed with SC red cells.
7. 1:10 dilution of serum from SC* sick birds.

Groups (i) and (ii) differ significantly, $P < 0.12$
Groups (i) and (iii) differ significantly, $P < 0.02$
Groups (a) and (b) differ significantly, $P < 0.02$

majority (83%) of these birds. On autopsy extensive lymphomas were found. A detailed description of the immunological and pathological events occuring in these category of birds is given in Table 21.

Table 21.

Sequence of immunological and pathological events in a minority (16%) of 15-day chimaeras and in the majority (83%) of 17-day chimaeras.

---

1. Early development of specific anti-donor strain antibody.

2. Young chimaeric birds (up to 12 - 16 weeks of age) which synthesize anti-donor strain antibody, have no detectable antibodies on their red cells. Their lymphocytes are specifically unresponsive for GVH reactivity.

3. After the 4th month of age these birds appear to become anaemic as judged by the decolourization of their comb.

4. At this stage both their red and white cells are coated by antibodies (see Table 19.A). Sera from these birds contain antibodies directed against both host and donor type antigens (see Table 19.B).

5. When heavily diseased birds are bled their peripheral cells show macroscopic signs of agglutination and microscopic examination reveals that large cells resembling erythroblasts are present in their peripheral blood.

6. Chimaerism was undetectable at this late stage of the disease and the ability of lymphocytes from these birds to cause GVH, CAM-pock reactions was generally suppressed as judged by the lack of reactivity observed when these lymphocytes were tested against outbred embryos.

7. 100% of the diseased birds die by the 10th month of age. On autopsy, spleen atrophy and severe lymphoid malignacy was diagnosed in the majority of these birds. The lymphoid malignacy shows the main features of lymphoid leucosis. The following types of tumours were found:

a. Large bursal tumours. Histological examination showed intrafollicular lymphoreticular infiltrations.

b. Metastasis to mesenteric lymphoid tissue.

c. Large metastatic nodules on the liver. Histological examination showed numerous masses of heterophiles, and large mononuclear cells with abundant cytoplasm.

d. Testicular tumours.

e. Infiltration of the lungs.

f. Numerous tumours in the kidneys.

g. Some of the birds developed ostopetrosis with involvement of both tarsal metatarsi and tibio tarsi.

The immunological and pathological characteristics shown by the majority of chimaeras generated at day 17 of embryonic development are also shown by a minority (16%) of

chimaeras generated at day 15 of embryogenesis. 8 out of a total of 36 birds were found to have anti-donor strain antibodies. Of these 8 birds, 6 succumbed to anaemia and lymphomas. In contrast, antibodies were never found in chimaeras generated at day 12 by parabiosis. The survival curves for the three different categories of chimaeras generated at days 12, 15 and 17 of embryonic development are shown in Fig. 6.

C. Summary

In this section, the immunological and pathological characteristics of chimaeric birds generated at day 17 of embryogenesis are described. These birds are unresponsive for GVH reactivity although they are producing anti-donor strain, B region, alloantibodies which are detected in their serum by the third week of age. With time they develop severe antibody-mediated autoimmune haemolytic anaemia. 83% of the diseased birds succumb to massive bursal and lymphoid tumours by the tenth month of age.

3. <u>Chimaeras generated at hatching (day 21 of ontogenic development)</u>

A. Establishment and estimation of chimaerism.

Chimaeras were produced at day 21 of ontogenic development, by intraperitoneal injection of allogeneic stem cells, immediately after hatching. Two different groups of chimaeras were produced. Firstly, 12 SC birds chimaeric with FP red cells were produced by injection of 15 day old

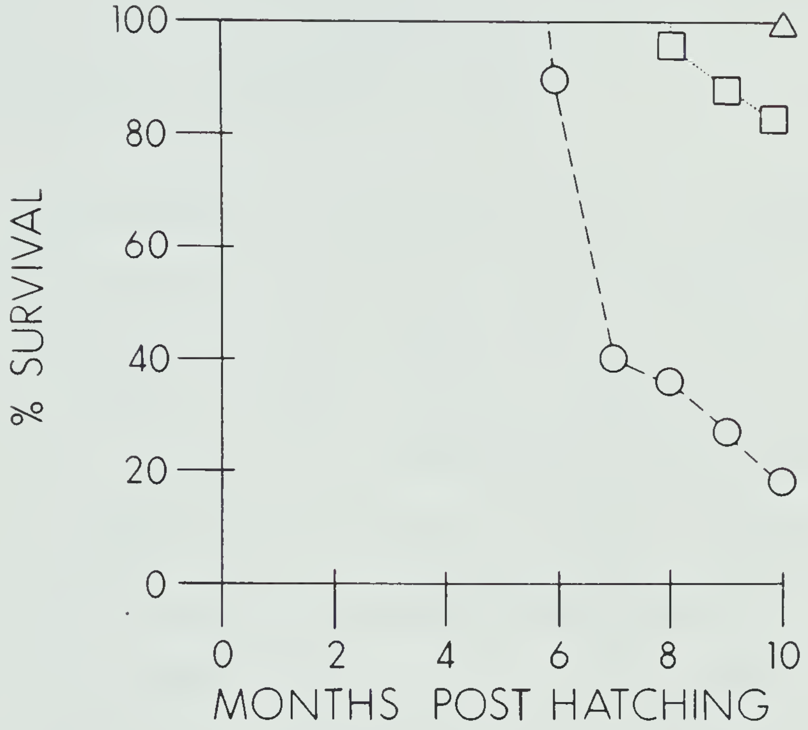

Fig. 6. Dependence of survival of chimaeric birds on the age at which chimaerism was induced. Curves are shown for 3 groups of SC birds made chimaeric with allogeneic stem cells at three different stages of embryonic development. Open traingles, represent 8 SC* birds made chimaeric by parabiosis at day 12 of embryogenesis. Open squares represent 36 SC* birds made chimaeric at day 15 of embryogenesis by I.V. injection of 15-day-old embryogenic FP spleen cells. Open circles represent 22 SC* birds made chimaeric at day 17 of embryogenesis by I.V. injection of age-matched allogeneic FP stem cells.

embryonic stem cells of splenic origin. Secondly, 10 SC birds chimaeric with FP red cells were produced by injection of 21 day old splenic stem cells. In both groups successful chimaerism ranging from 10 - 30% was achieved in the majority of birds (85%) when determined at three weeks of age. Chimaerism was estimated by both red cell agglutination and the cellular radioimmunoassay.

B. Immunological characteristics

The immunological properties of 7, randomly chosen, chimaeric birds were tested at two different ages. When tested at 3 weeks of age, 85% of these birds were chimaeric. Only one out of 22 birds tested contained anti-donor strain antibodies. Lymphocytes from all birds tested had normal levels of GVH reactivity when tested against host embryos of the donor type. When tested at the 4th month of age, these birds no longer had detectable chimaerism and had normal levels of GVH reactivity. The results from this group of experimental birds are presented in Table 22. These birds remained healthy throughout the examination period. They were terminated at the age of 6 months.

C. Summary

Chimaeras were generated at day 21 of ontogenic development by intraperitoneal injection of allogeneic splenic stem cells from either 15 day-old embryos or newly hatched chicks. These birds, although chimaeric, displayed normal GVH reactivity against donor type antigens when

Table 22. Immunological characteristics of chimaeras generated at day 21 of embryogenesis.[1]

	Properties examined at:					
	3 weeks of age			16 weeks of age		
Source of donor lymphocytes	% chimaerism[2]	CAM-pocks (mean ± SE) formed on FP hosts[3]	agglutination titers measured on FP red cells	% chimaerism	CAM-pocks (mean ± SE) formed on FP hosts[4]	Direct agglutination titers measured on FP red cells
SC normal	0	54 ±11.3	0	0	53.2±	0
SC* #1	30	N.T.**	0	0	43.1±	0
" #2	30	N.T.	0	0	53.2±	0
" #3	N.D.*	36.3± 8.0	1/4+	0	45.4±	0
" #4	20	43.1± 5.3	0	0	42.2±	0
" #5	10	44.6± 8.6	0	0	45.7±	0
" #6	30	56.2± 7.5	0	0	N.T.	0
" #7	20	N.T.	0	0	48.3±	0

[1]. SC chimaeras were generated at hatching by intraperitoneal injection of spleen cells either from 15 day old FP embryos or from 21 day-old newly hatched chicks.

[2]. Chimaerism was estimated by both red cell agglutination and by the radioimmunoassay as described in Materials and Methods.

[3]. The ability of 10^6PBLs to cause GVH reactions was measured by counting pocks on the CAM of FP embryos 4 days after inoculation.

[4]. The ability of 1:3 washed and reconstituted whole blood to cause GVH reactions was measured by counting pocks on the CAM of FP emrbyos 4 days after inoculation.

* N.D. = Not detectable

** N.T. = Not tested

tested at 3 weeks of age. The same birds, when tested at 16 weeks of age did not have detectable chimaerism. Again, normal values of GVH reactivity were found at this age. The birds remained healthy until they were deliberately sacrificed.

IV. Discussion

The main aim of the work presented in this thesis was to obtain an experimental system of unresponsiveness that could serve as a model for analyzing the cellular basis of self tolerance. Two properties of such a system were considered essential. i) The antigen should be administered early in the ontogenic development of the animal before the lymphopoietic system develops immunocompetence, and ii) the antigen concentration should be continuously maintained at high levels throughout the life of the individual once tolerance is established. The system employed possessed these two properties. Tolerance to MHC encoded alloantigens of the chicken was studied in haematopoietic chimaeras generated at early stages of embryogenesis. The self renewal capacity of the administered allogeneic stem cells ensured their continuous presence for the life span of the birds. The accessibility of the embryonic circulation of the chicken embryo and the technique of parabiosis allowed the antigen to be administered early in development.

The thymus becomes lymphoid at day 12 of embryogenesis (Venzke, 1952). All of the chimaeras produced by parabiosis at this time, and the majority of the chimaeras (83%) produced at day 15 of embryonic development, remained healthy, specifically unresponsive and chimaeric for their entire life span. By these criteria the immunological status

of these birds with respect to donor antigens is equivalent to that of a normal animal with respect to its own antigens. The study of the immunological status of these birds covered two broad classes of immunity. Firstly, they were completely unresponsive at the cell-mediated level, as measured by two manifestations of GVH reactivity, and secondly they were generally unresponsive at the humoral level in that they could not respond to donor antigens as measured by both a direct and indirect red cell agglutination assay. The results presented show that complete unresponsiveness was induced for all classes of immunity studied. The absence of indirect anti-donor haemagglutinating antibodies in these chimaeras was of particular interest. It has been recently reported that the sera of normal adult chickens contain natural antibodies against the MHC encoded antigens of the species with the exception of their own antigens. In this respect the immunological properties of these chimaeras with respect to donor antigens were identical to those of normal birds with respect to their MHC antigens. The cellular basis of unresponsiveness displayed by these chimaeras was investigated. The experiments performed took into consideration the main schools of thought on the nature of the cellular basis of tolerance to self antigens. These mechanisms were extensively discussed in the introductory chapter. In summary, they can be classified under two categories. The first is that self tolerance is established at an early stage of immunological development by

inactivation of the relevant clones on contact with antigen. The continuous presence of self antigens assures the continuous deletion of the clones as soon as they emerge throughout adulthood, thereby preventing the accumulation of many clones able to cooperate and be induced by self antigens. The second school of thought maintains that many clones specific for self antigens exist but are prevented from being induced by a mechanism of active suppression. The proposed active mechanisms can be divided into two categories, those in which: i) self tolerance is maintained by a class of humoral antibody that serves to protect the self antigens from recognition and destruction by more effective classes of immunity. Alternatively, it has been proposed that such antibodies can form complexes with self antigens that block the induction of anti-self immunocompetent cells; ii) self tolerance is maintained by a class of thymus derived suppressor cells. These cells can suppress the induction of anti-self clones by being specific either for the self antigen or idiotypes present on receptors with anti-self reactivity. This latter class of suppressor cell would most probably require the induction of anti-self reactivity in order that the idiotypes exist at sufficient concentration to induce suppressor cells (Jerne, 1973; Richter, 1975).

The results presented in this thesis argue in favor of a deletion mechanism. The arguments supportive of this conclusion are all indirect, in the sense that the

experiments performed failed to reveal any active mechanism in maintaining the unresponsive state. It should be pointed out, however, that the attempts to reveal anti-donor activity or inhibitory mechanisms were based on procedures that had revealed such activities or mechanisms in other experimental systems of unresponsiveness. The discovery of such reactivities in these systems served as the basis for postulating the existence of suppressive mechanisms that regulate anti-self reactivity. A brief summary of these experiments follows:

a) GVH unresponsiveness was not reversed after lymphocytes from unresponsive chimaeric birds were incubated in-vitro or treated with trypsin and/or phytohaemagglutinin prior to incubation. All these treatments have been shown to reveal immunological reactivity by lymphocytes from unresponsive animals (McGregor et al, 1967; Diener and Feldmann 1972; Waterfield et al, 1978). The difference between these systems and the system presented in this thesis is the time during ontogenic development at which tolerance was induced. In this group of chimaeras tolerance was induced at days 12 and 15 of embryogenesis. In the systems where unresponsiveness could be reversed, the unresponsive state was induced either at birth or by incubating lymphocytes from adult animals in-vitro.

b) The tolerant state of the chimaeric birds was not infectious. Neither humoral factors nor lymphocytes from

tolerant birds were able to inhibit the immunological reactivity of normal lymphocytes. Both humoral "blocking" factors and suppressor T cells have been demonstrated in different systems (Voisin et al, 1968, 1971; Hellstrom et al, 1971; Dorsch and Roser 1975, 1977; Holay et al, 1978; Gorczynski and McRae 1979 a, 1979 b). Again the difference between these systems and the system presented here is the time during development that unresponsiveness is induced. In all those cases where blocking factors or suppressor cells have been detected in transplantation systems the unresponsive state was induced at birth or later whereas the tolerance described here was induced at an earlier stage of development. There is one exception to the above generalisation, however, that should be discussed here. This is the observation that some of the tetraparental mice, made chimaeric by fusion at the "8 cell" stage, contain in their serum "blocking" antibodies. (Wegmann et al, 1971, Philips et al, 1971). This observation appears inconsistent with the thesis that tolerance induced at an early developmental stage results in complete unresponsiveness at both the cellular and humoral level. This finding may be explicable on the basis that the ratio of the two cell types changes with time in tetraparental mice (Mintz, 1969). It is possible that at a critical stage of development, and after the animal is immunocompetent, the concentration of a surface antigen present on one of the two types of lymphocytes becomes sufficient to induce an immune response

against it. The immune system would regard such an antigen in the same way as it regards neoantigens on malignant cells, that is it would react to it as foreign. The author believes that the same explanation applies where high levels of cell-mediated and humoral immunity are observed against self antigens present on broken-down mouse erythrocytes. These responses have been reported to appear at one week of age (Ramshaw and Eidinger 1977, Steele and Cunningham 1978; Cunningham, 1976).

c) Attempts to reverse the tolerant state by an in-vivo allogeneic effect were unsuccessful. The chimaeric birds, when challenged with a relatively low number of adult immunocompetent donor type lymphocytes, succumb to GVH death after 3 weeks. A similar experiment, performed in rats made neonatally tolerant to sheep erythrocytes, reversed the unresponsive state (McCullough, 1972).

d) Finally, the experiments in which the immunological properties of normal and chimaeric birds are compared, at the time when normal birds first showed immunocompetence, are difficult to reconcile with most models in which antiself reactivity is inhibited by anti-idiotypic cells. Most idiotype network theories require the induction of lymphocytes bearing the idiotype before anti-idiotypic cells can be induced (Jerne, 1973; Richter, 1975). This leads to the prediction that should anti-idiotypic suppressor cells or antibodies be responsible for the inactivation of anti-

self responses an initial phase of anti-self reactivity should be detectable. The results show that normal birds do not show any anti-self reactivity and that birds made chimaeric at day 15 did not react against self or donor antigens when tested at the earliest time of immunocompetence. Tolerance, induced during embryogenesis, was evident at the time of hatching when the first GVH immunocompetent lymphocytes can be detected after polyclonal activation.

The observation made here that the administration of antigen early in embryogenesis results in tolerance at both the cell-mediated and humoral level is in agreement with other observations in which tolerance to alloantigens or protein antigens is induced during embryogenesis (Hasek, 1953; Turk and Humphrey 1961; Humphrey and Turk, 1961; Parish and Lieu, 1972, Waters et al., 1979).

The importance of the continuous presence of the antigen at an appropriate concentration for maintaining the tolerant state was shown in a group of birds where chimaerism was not induced. These birds, although completely tolerant as young adults, spontaneously lost tolerance with age.

Chimaeras produced at a later stage of embryonic development that is at day 17, displayed specific unresponsiveness for GVH reactivity but a high proportion of them contained anti-donor strain antibodies in their sera.

The possibility that these antibodies are responsible for the lack of GVH reactivity was extensively tested. It was not possible to detect any inhibitory activity of these sera on the GVH reactivity of normal lymphocytes. Critical experiments to determine whether inhibitory cells are responsible for the lack of GVH reactivity were not performed. Extensive bleeding of these birds was avoided as it was important to examine their immunological and pathological condition over their entire life time. Nevertheless, there was an indication in 5 different experiments that lymphocytes from GVH unresponsive birds, containing anti-donor strain antibody, have inhibitory cells that suppress the GVH, CAM-POCK reactivity of normal lymphocytes. These suppressor cells are found in an experimental situation resembling an autoimmune state. They may be analogous to those found in mice which mount an anti-self red cell response after repeated challenge with rat erythrocytes (Cooke et al., 1978). This is an area of investigation which would be worthwhile pursueing.

Most of the immunological properties of chimaeras, produced at day 17 of embryogenesis, are the same as those of mice and rats made unresponsive to transplantation antigens by administering antigen at birth. There are several reports that a proportion of these animals contain blocking antibodies and suppressor cells which inhibit the induction of cell-mediated cytotoxicity mounted by normal lymphocytes (Voisin et al., 1968; Gorczynski and McRae

1979d; 1969b; Holan et al., 1978). There is a major difference between the unresponsiveness to transplantation antigens induced in mice and rats at birth and that induced in chickens at day 17 of embryogenesis. A chimaeric state was never detected in the first systems, whereas a high level of chimaerism is displayed by these chickens. The high degree of chimaerism may lead to chronic stimulation and be necessary for the development of the observed autoimmunity.

The finding that there are critical stages of embryonic development during which either tolerance or immunity can be induced is not new. Buxton reported in 1954, that the administration of killed Salmonella pullorum to chicken embryos leads to tolerance if the antigen is administered before the 16th day of embryogenesis. Administration of the antigen at later times of embryogenenis did not induce tolerance. The same conclusion was reached by Mitchison using turkey erythrocytes (Mitchison, 1962). Both authors accounted for the importance of the time at which antigen is administered as being due to the transfer of antibodies from the yolk sac to the embryonic circulation which occurs on the 15th day of incubation. There are similar observations in other species. Attempts to induce transplantation tolerance in mice at birth were successful in only 8% of the animals whereas 23% and 43% of the mice were tolerant when antigen was given at days 18 and 15 of gestation respectively (Billingham et al, 1956). This is not surprising in view of the fact that cytotoxic killer

precursor cells can be detected in the spleens of two day old mice (Pilarski, 1977; Wu, 1978).

The second important but unexpected finding was the development of autoimmune disease and malignacy in the chimaeras generated at day 17 of embryogenesis. These birds are highly chimaeric as young adults and contain anti-donor strain antibodies. These antibodies are ineffective at eliminating chimaerism. It seems likely that the continuous presence of donor type cells in high proportion stimulates the production of anti-host antibodies possibly due to the presence of donor antigens that cross react with the host. This process could lead to severe antibody-mediated haemolytic anaemia. Examples are reported in the literature in which autoimmunity can be induced by chronically immunizing with antigens that crossreact with self. For example, autoimmunity is known to be induced in mice after prolonged immunization with xenogneic RBCs (Cox and Keast, 1973) or with malaria-infected syngeneic RBCs (Rosenberg, 1978).

The autoimmune birds described in this thesis develop massive lymphoid tumors. The association between generalized autoimmunity and lymphoid neoplasms also exists in NZB mice. These mice develop autoantibodies against thymus cells when they are one month old, (Shirai and Mellors, 1971), and subsequently develop anti-red cell, anti-DNA, anti-RNA and anti-G antibodies (Gross-type murine leukemia virus related

antigen). 25% of these mice develop lymphoid neoplasia (Schwartz and Schwartz, 1968). It appears that the experimental system described in chickens resembles in many ways the naturally occuring autoimmune disease in N2B mice. It seems to me that it would be very interesting to determine whether the autoimmune state could be prevented in these mice by inducing tolerance before birth to the antigens present on adult thymocytes. There are two experimental observations that I was able to find that confirm the observations reported above, namely that autoimmunity and malignacy can be experimentally induced during embryogenesis. In 1956, Owen reported that R.H. Ripley had observed that chimaeric rats produced by embryonic administration of allogeneic fetal liver cells, succumb to liver tumors. More recently, it was reported tha 4 out of 27 Dove chimaeras, produced at day 8 of embryogenesis by parabiosis (the total gestation period being 14 days), developed antibodies against antigens of one of the partners. The red cells of 2 of these 4 birds were coated with antibodies. There is no report on the cause or nature of their death (McGary et al., 1975). The lymphomas which were observed in the current work were similar to those of avian leucosis which is a neoplasm of the B cell system induced by an RNA tumor virus in chickens (Petersoy et al., 1964). It is possible that chronic stimulation of the B cell system led to the activation of the virus and the induction of lymphomas.

Finally, these studies showed that attempts to induce unresponsiveness at hatching failed although successful chimaerism was temporarily produced. It seems that at this stage of ontogenic development chickens behave as adults since they are able to mount an effective cell-mediated immune response and actively reject the foreign tissue.

Table 23. Immunological and pathological characteristics of Avian-haematopoeitic chimaeras generated at different stages of embryonic development.

			Proportion of bird with:		
Chimaeras made at day:	% chimea-rism	No of birds tested	Specific anti-donor GVH	Specific anti-donor antibody	Haemolytic anaemia, lymphomas
12, by paraboisis	10-40	8	0/8	0/8	0/8
15, by I.V. injection of 15-day-old allogeneic embryonic stem cells	5-40	36	0/36	8/36	6/36
17, by I.V. injection of 17-day-old allogeneic embryonic stem cells	5-30	22	0/22	13/22	18/22
21, by I.P. injection of 15-day-old allogeneic embryonic stem cells	10-30 at 3 weeks 0 at >16 weeks	12	12/12	1/12	0/12

The results obtained in this thesis are summarized in Table 23. The main conclusions are:

1) Chimaeras generated at an early stage of embryonic development remain healthy, specifically unresponsive and chimaeric during a three year examination period. Unresponsiveness was observed at both the cell-mediated and humoral level and there was no evidence that it was mediated by active suppression. 2) Chimaeras generated at a later stage of embryonic development display unresponsiveness at the cell-mediated level but they produce specific anti-donor strain antibody by the third week post hatching. These chimaeras develop severe antibody-mediated haemolytic anaemia by the sixth month of age. Eighty-three percent of the autoimmune birds succumbed to massive bursal and other lymphoid tumors by the tenth month of age. 3) Attempts to induce unresponsiveness at hatching failed. Chimaeras generated at that stage of development displayed normal levels of cell-mediated immunity. These birds eliminated chimaerism by the third month of age.

An active inhibitory mechanism mediating the tolerant state, in the chimaeras generated at early stages of development, could not be demonstrated. It can be argued that this is due to limitations in the methodology employed. Nevertheless, these experiments demonstrate that there are clear cut immunological and long term pathological differences between the chimaeras generated at early and

late stages of development. The observations made provide a basis for explaining the conflicting results reported in the literature when apparently similar systems of unresponsiveness to transplantation antigens are analysed.

An interesting observation was made during the ontogenic studies of GVH immunocompetence which is not directly related to the main concern of these studies. Spleen cells from young chicks can initiate the development of "lymphoid-like colonies" on the CAM of chicken embryos. Experiments show that the size of these colonies depends on whether lymphocytes were inoculated onto syngeneic or allogeneic hosts. The observation that large white colonies are initiated only on allogeneic hosts suggested a possible interaction between immunocompetent lymphocytes and lymphoid stem cells. This interpretation was supported by the observation that the size of the large colonies is reduced when allogeneic cells from tolerant birds were incubated on donor type host-embryos. These observations require confirmation and they might serve as basis for further experimentation.

The picture that emerges from the work presented in this thesis would be confirmed and enlarged by further work. The nature of the suppressor cells, present in day 17 chimaeras, is unknown. It would be of interest to determine whether they are specific in their suppression of host anti-donor GVH reactivity, make attempts to determine what their

biological role is and why the presence of anti-donor antibodies does not eliminate chimaerism. The nature of the immunological and pathological characteristics of day 17 chimaeras resembles those of certain naturally-occuring diseases involving autoimmunity and neoplasms of the immune system. It would be of considerable interest to ascertain whether such diseases are initiated by an "internal" host versus graft reaction as the properties of the day 17 chimaeras might suggest. This could be approached by determining whether adults, that display similar immunological and pathological characteristics as these chimaeras, have viral or other antigens present on their haematopoietic and or lymphoid tissue that are not present in the developing embryo and to which the animal is not tolerant. Should this be the case, it would be worthwhile to determine whether the disease could be prevented by inducing tolerance to these antigens at early stages of embryonic development.

BIBLIOGRAPHY

Ada, G.L.,: Antigen binding cells in tolerance and immunity. Transplant. Rev. 5:105-129 (1970).

Ada, G.L., and M.G. Cooper: The in-vivo localization patterns and the in-vitro binding of lymphocytes of normal and tolerant rats by Salmonella flagellin and its derivatives. Ann. N.Y. Acad. Sciences. 181:96-107 (1971).

Allison, A.C., and A.M. Denmann: Self-tolerance and autoimmunity. Brit. Med. Bull. 31:124-128 (1976).

Asherson, G.L.: Selective and specific inhibition of 14 hour skin reactions in the guinea pig. Immunology. 10:179-186 (1966).

Atkins, R.C., and W.L. Ford: The effect of lymphocytes and serum from tolerant rats on the GVH activity of normal lymphocytes. Transplantation. 13:442-444 (1972).

Bankhurst, A., G. Torrigiani, and A.C. Allison: Lymphocytes binding Human thyroglobulin in healthy people and its relevance to tolerance for autoantigens. Lancet. 1:226-230 (1973).

Barker, C.F., and R.E. Billingham: Immunologically privileged sites. Adv. Immunol. 25:1-54 (1977).

Barnes, R.D., and C.G. Graham: Tolerance in early embryo aggregation-derived mouse chimaeras. Cell. Immunol. 21:146-152 (1976).

Benjamin, D.C.: Evidence for specific suppression in the maintenance of immunologic tolerance. J. exp. Med. 141:635-646 (1975).

Benjamin, D.C.: Neonatally induced tolerance to HGG: Duration in B cells and absence of specific suppressor cells. J. Immunol. 119:311-314 (1977).

Bernstein, I.D., B.L. Hamilton, P.W. Wright, R. Burstein and K.E. Hellstrom: Mixed lymphocyte culture reactivity in operationally tolerant rats. Relationship of lymphocyte-mediated reactivity and serum-blocking activity. J. Immunol. 114:320-325 (1975).

Beverly, P.C., L. Brent, C.G. Brooks, P.B. Medawar, and E. Simpson: In-vitro reactivity of lymphoid cells from tolerant mice. Transplant. Proc. 5:679-684 (1973).

Billingham, R.E.: The induction of tolerance of homologous

tissue grafts. Transplantation of tissues and cells. ed. R.E. Billingham and W.K. Silvers. p. 87-106 (1961).

Billingham, R.E., and T. Boswell: Studies on the problem of corneal homografts. Proc. Roy. Soc. s.B. 141:392-406 (1953).

Billingham, R.E., and L. Brent: Quantitative studies on tissue transplantation immunity. IV. Induction of tolerance in newborn mice and studies on the phenomenon of runt disease. Phil. Trans. Roy. Soc. 242:439-477 (1959).

Billingham, R.E., and W.K. Silvers: Induction of tolerance of skin isografts from male donors in female mice. Science. 128:780-781 (1958).

Billingham, R.E., and W.K. Silvers: Studies on tolerance of the Y chromosome antigen in mice. J. Immunol. 85:14-26 (1960).

Bilingham, R.E., L. Brent, and P.B. Medawar: Actively acquired tolerance of foreign cells. Nature. 172:603-606 (1953).

Billingham, R.E., L. Brent, and P.B. Medawar: Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Roy. Soc. London. Phil. Trans. s.B. 239:357-412 (1956).

Billingham, R.E., L. Brent, and P.B. Medawar: Extraction of antigens causing transplantation immunity. Transplant. Bull. 5:377-381 (1958).

Billingham, R.E., W.K. Silvers, and D.B. Wilson: Further studies on adoptive transfer of sensitivity of skin homografts. J. exp. Med. 118:397-420 (1963).

Binz, H.: Local graft-versus-host reaction in mice specifically inhibited by anti-receptor antibodies. Scand. J. Immunol. 4:79-87 (1975).

Binz, H., and B. Askonas: Inhibition of mixed-leucocyte culture by anti-idiotypic antibodies. Europ. J. Immunol. 5:618-623 (1975).

Binz, H., and H. Wigzell: Successful induction of specific tolerance to transplantation antigens using auto-immunization against the recipients own natural antibodies. Nature. 262:294-295 (1976).

Binz, H., J. Lindenmann, and H. Wigzell: Inhibition of local graft-versus-host reaction by anti-alloantibodies. Nature. 246:146-148 (1973).

Blair, P.B., and M. Lane: Non T cell killing of mammary tumour cells by spleen cells: Secretion of antibody and recruitment of cells. J. Immunol. 15:184-189 (1975).

Boyse, E.A., E.M. Lance, E.A. Carswell, S. Cooper, and L.J. Old: Rejection of skin allografts by radiation chimaeras: Selective gene action in the specification of cell-surface structure. Nature. 227:901-903 (1970).

Breinl, F., and F. Haurowitz: Chemische untersuchungen des praezipitates aus haemoglobin und anti-haemoglobinserum und bernerkyngen uber die natur der antikoerpen. Hoppe-Seyler's Ztsch. Physiol. Chem. 192:45-57 (1930). Reviewed by F. Haurowitz in J. Cellular and Comp. Physiol. 47, Suppl. 1:1-16 (1956).

Brent, L., and T.H. Courtenay: On the induction of split tolerance. Mechanisms of immunological tolerance. ed. M. Hasek, A. Lengerova and M. Voftiskova. 113-121 (1961).

Bretscher, P.A., and M. Cohn: A theory of self-non-self discrimination. Science. 169:1042-1049 (1970).

Bretscher, P.A.: The control of humoral and associative antibody synthesis. Transplant. Rev. 11:217-267 (1972).

Briles, W.E., and W.H. McGibbon: Heterozygosity of inbred lines of chickens at two loci effecting cellular antigens. Genetics. 33:605, (1948).

Briles, W.E., H.R. Stone, and R.K. Kole: Marek's disease: Effects of B histocompatibility alloalleles in resistant and susceptible chicken lines. Science. 195:193-195 (1977).

Brooks, C.G.: Clonal inactivation in neonatal tolerance. Europ. J. Immunol. 5:747-752 (1975).

Brooks, C.G.: Induction of neonatal transplantation tolerance. Do MLC antigens provide the major barrier? Transplantation. 21:347-350 (1976).

Burnet, F.M., J.D. Stone and M. Edney: The failure of antibody production in the chick embryo. Aust. J. Exp. Biology 28:291-297 (1950).

Burnet, F.M.: The clonal selection theory of acquired immunity. Cambridge University Press (1959).

Burnet, F.M.: Immunological recognition of self. Science. 133:307-311 (1961).

Burnet, F.M., and F. Fenner: The production of Antibodies.

2nd ed., MacMillan, Melbourne and London.

Buxton, A.: On the transference of bacterial antibodies from the hen to the chick. J. Gen. Microbiol. 7:268-286 (1952).

Buxton, A.: Antibody production in ovarian embryos and young chicks. J. Gen. Microbiol. 10:398-410 (1954).

Cannon, J.A., and W.P. Longmire: Studies of successful skin homografts in the chicken. Ann. Surgery. 135:60-68 (1952).

Carter, B.G., and B. Cinader: Some experiments on acquired immunological tolerance in the goat. Ann. N.Y. Acad. Sci. 87:363-381 (1960).

Ceppelini, R.: Old and new facts and speculations about transplantation antigens of man. Progress in Immunology. ed. B. Amos. 973-1025 (1971).

Chase, M.W.: Immunological tolerance. Ann. Rev. Microbiol. 13: 349-376 (1959).

Chase, M.W., and J.R. Battisto: Mechanisms on hypersensitivity. Henry Ford Symposium. J.A. Churchill, London. p. 507-517 (1959).

Chou, G.T., S. Dubiski, and B. Cinader: Antibody formation and experimentally induced chimaerism in very young rabbits. Nature. 211:34-36 (1966).

Chutna, J., M. Hasek, V. Holan, and M. Sladerek: Induction of specific transplantation tolerance with blood serum in newborn and adult rats. Europ. J. Immunol. 7:298-301 (1977).

Cinader, B., and J.M. Dubert: Acquired immune tolerance to human albumin and the response to subsequent injections of diazo-human albumin. Brit. J. Exptl. Pathology. 36:515-529 (1955).

Cooke, A., P.R. Hutchings and J.H.L. Playfair: Suppressor T cells in experimental autoimmune haemolytic anaemia. Nature 273:154-155 (1978).

Cox, K.O. and D. Keast: Erythrocyte autoantibodies induced in mice immunized with rat erythrocytes. Immunol. 25:531-537 (1973).

Cunningham, A.J.: Large numbers of cells in normal mice produce antibody to components of isologous erythrocytes. Nature. 252:749-750 (1974).

Cunningham, A.J.: Self-tolerance maintained by active suppressor mechanisms. Transplant. Rev. 31:23-43 (1976).

Danforth, C.H., and F. Foster. SKin transplantation as a means of studying genetic and endocrine factors in the fowl. J. exp. Zoology. 52-53:443-470 (1929).

Diener, E., and M. Feldmann: Antibody-Mediated Suppression of the immune response in-vitro. J. exp. Med. 132:31-43 (1970).

Diener, E., and M. Feldmann: Mechanisms at the cellular level during induction of high zone tolerance in-vitro. Cell. Immunol. 5:130-136 (1972).

Diener, E., and C.A. Waters: Restraints on current concepts of Self Tolerance. J. Supramolec. Structure. ICN-UCLA Symposia Abst. No. 596 (1979).

Dietrich, F., and W.O. Weigh: Induction of tolerance to heterologous proteins and their catabolism in C57BL/6 mice. J. exp. Med. 117:621-631 (1963).

Dixon, F.J., and P.J. Maurer: Immunological unresponsiveness induced by protein antigens. J. exp. Med. 101:245-257 (1955).

Dorsch, S., and B. Roser: T cells mediate transplantation tolerance. Nature. 258:233-235 (1975).

Dorsch, S., and B. Roser: Recirculating suppressor T cells in transplantation tolerance. J. exp. Med. 145:1144-1157 (1977).

Doyle, M.D., D.E. Parks, and W.O. Weigle: Specific suppression of the immune response by HGG tolerant spleen cells. I. Parameters affecting the level of suppression. J. Immunol. 116:1640-1645 (1976).

Droege, W.: Amplifying and suppressive effect of thymus cells. Nature. 234:549-551 (1971).

Droege, W.: The antigen-inexperienced thymic suppressor cells: A class of lymphocytes in the young chicken thymus that inhibits antibody production and cell mediated immune responses. Europ. J. Immunol. 6:279-287 (1978).

Droege, W., and K. Mayor: Graft-versus-host reactivity and inhibitory serum factors in allograft tolerant chickens. Transplant. 19:517-520 (1975).

Ehrlich, P.: On immunity with special reference to cell

life. Proc. Roy. Soc. London. 66:424-448 (1900).

Eichmann, K.: Idiotype Suppression. I. Influence of the dose and of the effector functions of anti-idiotypic antibody on the production of an idiotype. Europ. J. Immunol. 4:296-302 (1974).

Eichmann, K., and K. Rajewsky: Induction of T and B cell immunity by anti-idiotypic antibody. Europ. J. Immunmol. 5:661-666 (1975).

Elkins, W.L.: Cellular control of lymphocytes initiating graft vs hosts reactions. Cell. Immunol. 4:192-196 (1972).

Elkins, W.L.: Deficit of specific thymus-dependent lymphocytes in transplantation tolerance in rats. J. exp. Med. 137:1097-1107 (1973a).

Elkins, W.L.: The cellular basis of transplantation tolerance. Transplant. Proc. 5:685-689 (1973b).

Elkins, W.L., J.S. Adams, D.H. Fox, D.B. Wilson, and F.P. Stuart: Partial tolerance and immunity after adoptive abrogation of transplantation tolerance in the rat. Cell. Immunol. 9:412-425 (1973).

Elson, C.J.: Tolerance in differentiating B lymphocytes. Europ. J. Immunol. 7:6-10 (1977).

Fitch, F.W., and H. Ramseier: Generation of cytotoxic T lymphocytes in-vitro. VIII. Failure of anti-RS to inhibit the generation of cytolytic T lymphocytes or cytolytic T lymphocyte activity. J. Immunol. 117:504-570 (1976).

Fravi, G., and J. Lindenmann: Induction by allogeneic extracts of liver-specific precipitating autoantibodies in the mouse. Nature. 218:141-143 (1968).

Fredericksen, T.L., B.M. Longenecker, F. Pazderka, D.G. Gilmour, and R.F. Ruth: A T cell antigen system of chickens: Ly-4 and Marek's disease. Immunogenetics. 5:535-552 (1977).

Friedman, H., and W.L. Gaby: Immunological unresponsiveness to Shigella antigens in chickens. J. Immunol. 84:441-448 (1960a).

Friedman, H., and W.L. Gacy: Immunological unresponsiveness in mice following neonatal exposure to Shigella antigens. J. Immunol. 85:478-482 (1960b).

Friedman, H.: Immunological tolerance to microbial antigens.

II. Suppressed antibody plaque formation to Shigella antigen by spleen cells from tolerant mice. J. Bacteriol. 92:820-827 (1966).

Gershon, R.K., and K. Kondo: Infectious immunological tolerance. Immunology. 21:903-914 (1971).

Globerson, A., and T. Umiel: Ontogeny of suppressor cells. Transplantation. 26:438-442 (1978).

Goldberg, E., E. Boyse, M.S. Scheid and D. Bennett: Production of H-Y antibody by female mice that fail to reject male skin. Nature New Biol. 238:55-57 (1972).

Gorczynski, R.M., S. MacRae, and J.E. Till: Analysis of mechanisms of maintenance of neonatally induced tolerance to foreign alloantigens. Scand. J. Immunol. 7:453-465 (1978).

Gorczynski, R.M., and S. MacRae: Suppression of cytotoxic response to histoincompatible cells. I. evidence for two types of T lymphocyte derived suppressor acting at different stages in the induction of a cytotoxic response. J. Immunol. 122:737-746 (1979a).

Gorczynski, R.M., and S. MacRae: Suppression of cytotoxic response to histoincompatible cells. Analysis of the role of two independent T suppressor pools in maintenance of neonatally induced allograft tolerance in mice. J. Immunol. 122:747-752 (1979).

Gowland, G., G. Hobbs, and H.D. Byers: Attempts to suppress agglutinin production to a bacterial antigen (Pseudomonas SP.) in the neonatal rabbit. J. Pathol. Bacteriol. 90:443-457 (1965).

Grebenau, M.D., S.P. Lerman, M.A. Paladino, and G.J. Thorbecke: Suppression of adoptive antibody responses by addition of spleen cells from aggamaglobulinaemic chickens "immunized" with histoincompatible bursa cells. Nature. 260:46-48 (1976).

Gunther, J. Balcarova, K. Hala, E. Rude, and T. Hraba" Evidence for an association between immune responsiveness of chicken to (T, G)A...L and the major histocompatibility system. Europ. J. Immunol. 4:548-553 (1974).

Hala, K., M. Hasek, M. Hlozanick, J. Horst, F. Knizerova, and H. Mervarovd: Syngeneic lines of chickens. II. Inbreeding and selection within M, W and I lines and crosses between the C, M and W lines. Folia Biol. (Praha) 12:407-422 (1966).

Hala, K., M. Vilhelmova, J. Hartmatova: Probable crossing-over in the B blood group system of chickens. Immunogenetics 3:97-103 (1976).

Hanau, R., and J. Oyama: Inhibition of antibody formation in mature rabbits by contact with the antigen at early age. J. Immunol. 73:49-53 (1954).

Hartmanova, J., K. Hala, M. Vilhelmova: Differences in the distribution of chicken B complex antigens demonstrated by means of tolerance. Folia Biol. (Praha). The XIV International Symposium on Laboratory Animals. Section 1. Avian Immunology. 23:406-407 (1977).

Hasek, M.: Parabiosis in birds during embryonic development. Ceskoslovenska Biol. 2:25-27 (1953).

Hasek, M.: Manifestations of vegetative approximation in the adaptation of higher animals to foreign antigens. Ceskoslovenska Biol. 3:327-334 (1954).

Hasek, M.: Tolerance phenomena in birds. Proc. Roy. Soc. London s.B. 146:67-77 (1957).

Hasek, M., A. Leugerova, and T. Hraba: Transplantation immunity and tolerance. Adv. Immunol. 1:1-66 (1961).

Hasek, M., F. Knizetova and H. Mervartova. Syngeneic lines in chickens. I. Inbreeding and selection by means of skin grafts and tests for erythocyte antigens in C line chickens. Folia Biol. (Praha) 12:335-342 (1966).

Hasek, M., J. Chutna, V. Holan, and S. Sladecek: Induction of transplantation tolerance using serum as antigen source. Nature. 262:295-296 (1976).

Haskova, V.: Transplantation immunity and immunological tolerance and the study of antigenicity of tissues and their alternatives. Biological Problems of Grafting p. 95-106 (1959).

Hellstrom, I., K.E. Hellstrom, C.A. Evans, G.H. Heppner, G.E. Pierce, and J.P.S. Yang: Serum-mediated protection of neoplastic cells from inhibition by lymphocytes immune to their tumour-specific antigens. Proc. Nat. Acad. Sci. Wash. 62:362-368 (1969).

Hellstrom, I., K.E. Hellstrom, and A.C. Allison: Neonatally induced allograft tolerance may be mediated by serum borne factors. Nature. 230:49-50 (1971).

Hellstrom, K., I. Hellstrom, and J. Brown: Abrogation of cellular immunity to antigenically foreign mouse embryonic cells by a serum factor. Nature. 244:914-915

(1969).

Hellstrom, K., and I. Hellstrom: The role of serum factors (blocking antibodies) as mediators of immunological non-reactivity to cellular antigens. CIBA Foundation Symposium. Ontogeny of acquired immunity. 133-143 (1971).

Heron, I.: Is transplantation tolerance in rats serum-mediated? Transplantation. 15:534-539 (1973).

Holan, V., J. Chutna, and M. Hasek: Participation of H-2 regions in neonatally induced transplantation tolerance. Immunogenetics. 6:397-401 (1978a).

Holan, V., J. Chutna, and M. Hasek: Specific suppression of antigen-reactive cells in neonatal transplantation tolerance. Nature. 274:895-897 (1978b).

Howard, J.G., and C. Hale: Lack of neonatal susceptibility to induction of tolerance by polysaccharide antigens. Europ. J. Immunol. 6:486-492 (1976).

Humphrey, J.H.: Immunological unresponsiveness to protein antigens in rabbits I. Duration of unresponsiveness following a single injection at birth. Immunology. 7:449-461 (1964).

Humphrey, J.H., and J.L. Turk: Immunological unresponsiveness in guinea pigs. I. Immunological unresponsiveness to heterologous serum proteins. Immunology. 4:301-309 (1961).

Jaffe, W.P., and E.M. McDermid: Blood groups and splenomegaly in chick embryos. Science. 137:984-984 (1962).

Janicki, B.W., G.P. Schechter, and K.E. Schultz: Cellular reactivity to tuberculin in immune and serologically unresponsive guinea pig. J. Immunol. 105:527-530 (1970).

Jerne, N.K.: The natural selection theory of antibody formation. Proc. Nat. Acad. Sci. Wash. 41:489-859 (1955).

Jerne, N.K.: The immune System. Sci. American. 229:52-60 (1973).

John, U., E. Carswell, E.A. Boyse, and G. Alexander: Production of O antibody by mice that fail to reject I-incompatible skin grafts. Nature New Biol. 238:57-58 (1972).

Joller, P.W.: Graft versus host reactivity of lymphoid cells inhibited by anti-recognition structure serum. Nature New Biol. 240:214-215 (1972).

Ivanyi, J., and J.G. Howard: Comparative aspects of tolerance to protein and polysaccharide antigens. Immunological tolerance to tissue antigens. ed. N.W. Nisbet, and M.W. Elves. p. 145-158 (1971).

Iverson, G.M., and J. Lindenmann: The role of carrier determinant and T cells in the induction of liver specific autoantibodies in the mouse. Europ. J. Immunol. 2:195-197 (1972).

Kaliss, N.: Immunological enhancement of tumour homografts in mice. Cancer Research. 18:992-1003 (1958).

Keller, G.M., B.M. Longenecker, and E. Diener: Hemopoetic colonies on the CAM. Induction of embryonic adherent, non hemopoetic spleen cells. J. Cell. Physiol. 1979 (in press).

Kerr, W.R., and M. Robertson: Passively acquired antibodies for Tridiomonas foetus in very young calves. J. Hyg. Cambridge. 52:253-263 (1954).

Kimura, A.K.: Inhibition of specific cell-mediated cytotoxicity by anti-T cell receptor antibody. J. Exp. Med. 139:888-901 (1974).

Kinsky, R., and N.A. Mitchison: Tolerance of skin induced by erythrocytes in poultry. Transplantation. 1:224-231 (1963).

Klein, J.: Biology of the mouse histocompatibility-2 complex. Springer-Verlag New York-Heidelberg-Berlin (1975).

Koch, C., and M. Simonsen: Immune response genes in chickens. Antibody response to TGAL and GT. Immunogenetics 5:161-170 (1977).

Lafferty, K.J., K.Z. Walker, R.G. Scollay, and V.A.A. Killby: Allogeneic interactions provide evidence for a novel class of immunological reactivity. Transplant. Rev. 12:198-228 (1972).

Lafferty, K.J., I.S. Misko, and M.A. Cooley: Allogeneic stimulation modulates the in-vitro response of T cells to transplantation antigens. Nature. 249:275-276 (1974).

Lake, P. and N.A. Mitchison: Regulatory mechanisms in the immune response to cell surface antigens. Cold Spring

Harbor Symposia: XLI 589-595 (1976).

Lance, E.M., E.A. Boyse, S. Cooper, and E.A. Carswell: Rejection of skin allografts by irradiation chimaeras: Evidence for skin-specific transplantation barrier. Transplant. Proc. 3:865-868 (1971).

Law, L.W., E. Appella and P.W. Wright: Immunological enhancement of allogeneic tumour growth with soluble histocompatibility antigens. Proc. Nat. Acad. Sci. Wash. 68:3078-3082 (1971).

Law, L.W., E. Appella, S. Strober, P.W., Wright and T. Fischetti: Induciton of immunological tolerance to soluble histocompatibility-2 antigens of mice. P.N.A.S. 69:1858-1862 (1972).

Law, L.W., E. Appella, J.M. Cohen: Reactivity of lymphoid cells from mice humorally tolerant to histocompatibility antigens. Nature New Biol. 246:174-176 (1973).

Law, L.W., E. Appella, S. Strober, P.W. Wright and T. Fischetti: Soluble transplantation antigens. Further studies of their tolerogenic properties. Transplantation. 18:487-495 (1974a).

Law, L.W., E. Appella, J.M. Cohen and P.W. Wright: Role of serum factors from tolerant mice in blocking and in immunological enhancement. Transplantation. 18:14-19 (1974b).

Lederberg, J.: Genes and antibodies. Science. V:129, 1649-1653 (1959).

Lindahl, K.F.: Antisera against recognition sites. Lack of effect on the mixed lymphocyte culture interaction. Europ. J. Immunol. 2:501-504 (1972).

Longenecker, M.B., S. Sheridan, G.R.J. Law, and R.F. Ruth: Measurement of graft-versus-host-reactions on the chorioallantoic membrane of the chicken. Transplantation. 9:544-557 (1970).

Longenecker, M.B., F. Pazderka, G.R.J. Law, and R.F. Ruth: Genetic control of graft-versus-host competence. Transplantation. 14:424-431 (1972).

Longenecker, M.B., F. Pazderka, G.R.J. Law, and R.F. Ruth: The graft-versus-host reaction to minor alloantigens. Cell. Immunol. 8:1-11 (1973).

Longenecker, M.B., F. Pazderka, J.S. Gavora, J.L. Spencer, and R.F. Ruth: Lymphoma induced to Herpesvirus:

Resistance associated with a major histocompatibility gene. Immunogenetics. 3:401-407 (1976).

Longenecker, B.M., B. Singh, M. Gallatin, and C. Havele: Histocompatibility typing by cellular radioimmunoassay. Immunogenetics. 7:201-211 (1978).

Lubaroff, D.M., and W.K. Silvers: The importance of chimaerism in maintaining tolerance of skin allografts in mice. J. Immunol. 111:65-71 (1978).

Lydyard, P.M., and J. Ivanyi: The role of opsonization in anti-thymocyte globulin - induced suppression of graft-versus-host reactions in chick embryo. Transplantaion. 17:400-404 (1974).

McCullach, P.J.: The transfer of immunological competence to rats tolerant of sheep erythrocytes with lymphocytes from normal rats. Aust. J. Exp. Biol. MEd. Sci. 48:351-367 (1970a).

McCullach, P.J.: The immunological capacity of lymphocytes from normal donors after their transfer to rats tolerant of sheep erythrocytes. Aust. J. Exp. Biol. Med. Sci. 48:368-379 (1970b).

McCullach, P.J.: The abrogation of immunological tolerance by means of allogeneic confrontation. Transplant. Rev. 12:180-197 (1972).

McGary D.A., M. Nickells, G. Justines and M.R. Irwin: Probable loss of Tolerance and Stimulation of Autoimmunization in induced chimaeras in Doves. Immunogenet. 2:175-182 (1975).

McGregor, D.D., P.J. McCullach and J.L. Gowans: The role of lymphocytes in antibody formation. I. Restoration of the haemolysin response in X-irradiated rats with lymphocytes from normal and immunologically tolerant donors. Proc. Roy. Soc. London s.B. 168:229-243 (1967).

Meo, T., T. Matsunaga, and A.M. Rijnbeek: On the mechanism of self-tolerance in embryofusion chimaeras. Transplant. Proc. 5:1607-1610 (1973).

Metcalf, E.S., and N.R. Klinman: In-vitro tolerance induction of neonatal murine B cells. J. Exp. Med. 143:1327-1340 (1976).

Metcalf, E.S., A.F. Schrater, and N.R. Klinman: Murine models of tolerance induction in developing and mature B cells. Immunol. Rev. 43:143-183 (1979).

Miggiano, W.C., J. Birgen, and J.R.L. Pink: The mixed

lymphocyte reaction in chickens. Evidence for control by the major histocompatibility complex. Europ. J. Immunol. 4:397-401 (1974).

Miller, R.G., and H. Derry: A cell population in nu/nu spleen can prevent generation of cytotoxic lymphocytes by normal spleen cells against self antigens of the nu/nu spleen. J. Immunol. 122:1502-1519 (1979).

Mintz, B.: Experimental study of the developing mammalian egg: Removal of the zona pellucida. Science. 138:594-595 (1962).

Mintz, B.: Gene expression in allophenic mice. Control mechanisms in the expression of cellular phenotypes. ed. H.A. Padykula. p. 15-42 (1970).

Mintz, B., and W.K. Silvers: "Intrinsic" immunological tolerance in allophenic mice. Science. 158:1484-1487 (1967).

Mintz, B., and J. Palm: Gene control of hematopoiesis. I. Erythrocyte mosaicism and permanent immunological tolerance in allophenic mice. J. Exp. Med. 128:1013-1027 (1967).

Mitchison, N.A.: Blood transfusion in fowl. An example of immunological tolerance requiring the persistence of antigen. Biological problems of grafting (Symposium). p. 239-255 (1959).

Mitchison, N.A.: Tolerance of erythrocytes in poultry. Induction and specificity. Immunology. 5:341-358 (1962).

Mitchisson, N.A.: New ideas about self-tolerance and autoimmunity. Clinics in Rheumatic Diseases. 4/3:539-548 (1978).

Moller, G.: Mechanism of B-cell activation and self-non-self discrimination. Cold Spring Harbor Symposia. XLI:217-226 (1976).

Moller, G., E. Gronowicz, U. Person, A. Coutinho, E. Moller, L. Hammarstrom, and E. Smith: Spleen cells from animals tolerant to a thymus-dependent antigen can be activated by lipopolysaccharide to synthesize antibodies against the tolerogen. J. Exp. Med. 143:1429-1438 (1976).

Mosier, D.E., P.L. Cohen, and B.M. Johnson: Suppressor cells in the ontogeny of the immune response. Suppressor cells in immunity. ed. S.K. Sighal and N.R.St.C. Sinclair. p. 28-32 (1975).

Naor, D., and D. Sulitzneau: Binding of radioiodinated bovine serum albumin to mouse spleen cells. Nature. 214:687-688 (1967).

Naor, D., and D. Sulitzneau: Binding of ^{125}I-BSA to lymphoid cells of tolerant mice. Int. Arch. Allergy. 36:112-113 (1969).

Nossal, G.J., G.L. Ada, C.M. Austin: Antigens in immunity. X. Induction of immunological tolerance to salmonella adelaide flagellin. J. Immunol. 95:666-672 (1965).

Nossal, G.J., and B.L. Pike: Evidence for the clonal abortion theory of Blymphocyte tolerance. J. Exp. Med. 141:904-917 (1975).

Owen, R.D.: Immunogenetic consequences of vascular anastomosis between bovine twins. Science. 102:400-402 (1945).

Owen, R.D.: Erythrocyte antigens and tolerance phenomena. Proc. Roy. Soc. London. s.B. 146:8-18 (1957).

Parish, C.R.: Immune response to chemically modified flagellin. Evidence for a fundamental relationship between humoral and cell-mediated immunity. J. Exp. Med. 134:21-47 (1971).

Parish, C.R., and F.Y. Liew: Immune response to chemically modified flagellin. III. Enhanced cell-mediated immunity during high and low zone antibody tolerance to flagellin. J. Exp. Med. 135:298-311 (1972).

Pauling, L., and D. Cambell: The manufacture of antibodies in-vitro. J. Exp. Med. 76:211-220 (1942).

Pazderka, F., B.M. Longenecker, G.R.J. Law, and R.F. Ruth: The major histocompatibility complex of the chicken. Immunogenetics 2:101-128 (1975).

Pilarski, L.M.: Ontogeny of cell-mediated immunity. I. Early development of alloantigen-specific cytotoxic T-cell precursors in postnatal mice. J. exp. Med. 146:887-892 (1977).

Pilarski, L.M.: Antigen specific helper T cells are essential for cytotoxic T cell responses to metabolically inactivated stimulator cells. Europ. J. Immunol. 9:454-460 (1979).

Pink, J.R.L., and V.C. Miggiano: How chicken alloantisera inhibit the graft-versus-host and mixed lymphocyte reaction. Basel Institute for Immunology. Annual report. Abst. #63, p. 60 (1977).

Pink, J.R.L., W. Droege, K. Hala, V.C. Miggiano, and A. Ziegler: A three locus model for the chicken major histocompatibility complex. Immunogenetics 5:203-216 (1977).

Phillips, M., and T.G. Wegmann: Active suppression as a possible mechanism of tolerance in tetraparental mice. J. Exp. Med. 137:291-300 (1973).

Phillips, S.M., W.J. Martin, A.R. Shaw, and T.G. Wegmann: Serum mediated immunological non-reactivity between histoincompatible cells in tetraparental mice. Nature. 234:146-148 (1971).

Porter, K.A.: Runt disease and tolerance in rabbits. Nature. 185:789-790 (1960).

Qualtiere, L.F., and P.M. Meyers: A reexamination of humoral tolerance in chickens congenitally infected with an avian leucosis virus. J. Immunol. 122:825-829 (1979).

Ramsaw, I.A., and D. Eidinger: T-cell mediated immunity towards antigen(s) on isologous erythrocytes. Nature. 267:441-442 (1977).

Ramseier, H.: Immunization against abolition of transplantation tolerance. Europ. J. Immunol. 3:156-164 (1973).

Ramseier, H.: Antibodies to T cell receptors and to histocompatibility antigens. Cell. Immunol. 12:422-428 (1974).

Ramseier, H., and J. Lindenmann. Studies on antireceptor antibody and on alloantibody. Immunogenetics. 2:551-560. (1975).

Reichlin, M.: Localizing antigenic determinants in human haemoglobin with mutants. Molecular correlations of immunological tolerance. J. Mol. Biol. 64:485-496 (1972).

Richter, P.H.: A network theory of the immune system. Europ. J. Immunol. 5:350-354 (1975).

Robinson, J.H., and J.J.T. Owen: Transplantation tolerance induced in fetal mouse thymus in-vitro. Nature. 271:758-760 (1978).

Rollinghoff, M., and H. Wagner: Secondary cytotoxic allograft response in-vitro. I. Antigenic requirements. Europ. J. Immunol. 5:875-879 (1975).

Rosenberg, Y.J.: Autoimmune and polyclonal B cell responses

during murine malaria. Nature. 274:170-172 (1978).

Rouse, B.T., and N.L. Warner: The role of suppressor cells in avain allogeneic tolerance: Implications for the pathogenesis of Marek's disease. J. Immunol. 113:904-909 (1974).

Rowley, D.A., and F.W. Fitch: The mechanism of tolerance produced in rats to sheep erythrocytes. I. Plague-forming cell and antibody response to single and multiple injections of antigen. J. Exp. Med. 121:671-681 (1965).

Rubin, H., L. Fanshier, A. Cornelius, and W.F. Hughes: Tolerance and immunity in chickens after congenital and contact infection with an avain leucosis virus. Virology. 17:143-156 (1962).

Ryle, M.: Studies on possible serological blocks to species hybridization in poultry. J. Exp. Biol. 34:365-377 (1957).

Schechtman, A.M., and P.F. Knight: Transfer of proteins from the yolk to the chick embryo. Nature. 176:786-787 (1955).

Schied, M., E.A. Boyse, E.A. Carswell, and L.J. Old: Serologically demonstrable alloantigens of mouse epidermal cells. J. Exp. Med. 135:938-955 (1972).

Schierman, L.W., and A.W. Nordskog: Relationship of blood type to histocompatibility in chickens. Science. 134:1008-1009 (1961).

Schierman, L.W., and A.W. Nordskog: Influence of the B-blood group histocompatibility locus in chickens on a graft-versus-host reaction. Nature. 197:511-512 (1963).

Scwartz, J., J. Kletter, and A. Klopstack: Partial Immunological unresponsiveness to virus antigens in guinea pigs. Int. Arch. Allergy. 25:83-88 (1964).

Schwartz, R.H., C.G. Fathman, and D.H. Sachs. Inhibition of stimulation in murine mixed lymphocyte cultures with an alloantiserum directed against a shared Ia determiannt. J. Immunol. 116:929-935 (1976).

Schwarz, M.R.: The mixed lymphocyte reaction. An in-vitro test for tolerance. J. Exp. Med. 127:879-889 (1968).

Schwartz, R.S., and J. Andre-Schartz: Malignant lymphoproliferative diseases. Ann. Rev. Med. 19:269-282 (1968).

Scott, D.W., M. Veukataraman, and J.J. Jandinski: Multiple pathways of B lymphocyte tolerance. Immunol. Rev. 43:241-280 (1979).

Seamer, J.: The growth, reproduction and mortality of mice made immunologically tolerant to lymphocytic choriomeningitis virus by congenital injection. Arch. Fur. Die. Ges. Virus-Forschung. 15:169-177 (1965).

Sepalla, I., and K. Eichmann: Induction and characterization of Isogenic anti-idiotypic antibodies to Balb/c myeloma S117. Lack of reactivity with major idiotypic determinants. Europ. J. Immunol. 9:243-250 (1979).

Sercarz, E.E., and A.H. Coons: The absence of antibody-producing cells during unresponsiveness to BSA in the mouse. J. Immunol. 90:478-491 (1963).

Shirai, T., and Mellors, R.: Natural thymocytotoxic autoantibody and reactive antigen in NZB and other mice. Proc. Nat. Acad. Sci. Wash. 68:1412-1415 (1971).

Silvers, W.K.: The influence of removing lymphoid tissue on the persistence of tolerance of skin allografts in mice. J. Immunol. 113:804-809 (1974).

Simonsen, M.: Artificial production of immunological tolerance. Nature. 175:763-764 (1955).

Siskind, G.W., P.Y. Paterson, and L. Thomas: Induction of unresponsiveness and immunity in newborn and adult mice with pneumococual polysacharide. J. Immunol. 90:929-934 (1963).

Skopinka, E., and E. Skamene: Stimulation of DNA synthesis in mixed lymphocyte cultures of chickens. Folia Biol. (Praha) 14:296-298 (1968).

Smith, R.T.: Immunological tolerance of nonliving antigens. Adv. Immunol. 1:67-129 (1961).

Smith, R.T., and R.A. Bridges: Response of rabbits to defined antigens following neonatal injection. Transplant. Bull. 3/4:145-147 (1956).

Smith, R.T., and R.A. Bridges: Immunological unresponsiveness in rabbits produced by neonatal injection of defined antigens. J. Exp. Med. 108:227-250 (1958).

Staines, N.A., K. Guy, and D.A.L. Davies: The dominant role of Ia antibodies in the passive enhancement of H-2 incompatible skin grafts. Europ. J. Immunol. 5:782-789 (1975).

Steele, E.J., and A.J. Cunningham: High proportion of Ig-producing cells making autoantibody in normal mice. Nature. 274:483-484 (1978).

Streilein, J.W., and J. Klein. Neonatal tolerance induction across regions of H-2 complex. J. Immunol. 119:2147-2150 (1977).

Talmage, D.W.: Allergy and immunology. Ann. Rev. Med. 8:239-256 (1957).

Tarkowski, A.K.: Mouse chimaeras developed from fused eggs. Nature. 190:857-860 (1961).

Teale, J.M., J.E. Layton, and G.J.V. Nossal: In-vitro model for natural tolerance to self antigens. Inhibition of the development of surface-immunoglobulin-negative lymphocytes into T-dependent responsive B cells by antigen. J. Exp. Med. 150:205-217 (1979).

Tempelis, C.H., H.R. Wolfe, and A. Mueller: The production of immunological unresponsiveness by the intravenous injection of bovine serum albumin into the chick embryo. Brit. J. Exp. Pathol. 39:323-327 (1958).

Terres, G., and W.L. Hughes: Acquired immune tolerance in mice to crystalline bovine serum albumins. J. Immunol. 83:459-467 (1959).

Traub, E.: Epidemiology of lymphocytic choriomeningitis in a mouse stock observed for four years. J. Exp. Med. 69:801-817 (1939).

Triplett, E.L.: On the mechanism of immunologic self recognition. J. Immunol. 89:505-510 (1962).

Tucker, E.M., R.M. Moor, and L.E.A. Rowson: Tetraparental sheep chimaeras induced by blastomere transplantation. Immunology. 26:613-621 (1974).

Turk, J.L., and J.H. Humphrey: Immunological unresponsiveness in guinea pig. II. Effect of unresponsiveness on the development of delayed-type-hypersensitivity to protein antigens. Immunology. 4:310-317 (1961).

Venkataramau, M., and D. Scott: VI. Neonatal vs adult B cell tolerance: Differences in antigen-binding cell patterns and lipopolysaccharide stimulation. J. Immunol. 119:1879-1881 (1977).

Venzke, W.G.: Morphogenesis of the thymus of chick embryos. Am. J. Vet. Res. 13:395-402 (1952).

Vitetta, E.S., and J.W. Uhr: Immunoglobulin-receptors revisited. Science. 189:964-969 (1975).

Viosin, G.A., R.G. Kinsky, et J. Maillard: Reactivite immunitaire et anticorps facilitants chez des animaux tolerants aux homogreffes. Ann. Inst. Pasteur. Paris. 115:855-879 (1968).

Voisin, G.A., R.G. Kinsky, and H.T. Duc: Immune status of mice tolerant of living cells. II. Continuous presence and nature of facilitation-enhancing antibodies in tolerant animals. J. Exp. Med. 135:1185-1203 (1972).

Volkert, M., and J.H. Larsen: Immunological tolerance to viruses. Prog. Med. Virol. 7:160-207 (1965).

Wachtel, S.S.: H-Y antigens: Genetics and serology. Immunol. Rev. 33:33-58 (1977).

Wagner, H., G. Hammerling, and M. Rollinghoff: Enhanced in-vitro cytotoxic anti H-2 responses in the presence of Ia antigens. Immunogenetics. 2:257-268 (1975).

Warner, N.L., and L.A. Herzenberg: Tolerance and immunity to maternally derived incompatible IgG2a globulin in mice. J. Exp. Med. 132:440-447 (1970).

Waterfield, J.D., P.H. Bick, E. Moller, and G. Moller: Presence of alloantigen-reactive cells in neonatally tolerant mice. Scand. J. Immunol. 7:435-438 (1978).

Waters, C.A., L.M. Pilarski, T.G. Wegmann, and E. Diener: Tolerance induction during ontogeny. I. Presence of active suppression in mice rendered tolerant to human gamma globulin in utero correlates with the break down of the tolerant state. J. Exp. Med. 149:1134-1151 (1979).

Wegmann, T.G., and J.G. Gilman: Chimaerism for three genetic systems in tetraparental mice. Develop. Biol. 21:281-291 (1970).

Wegmann, T.G., I. Hellstrom, and K. Hellstrom: Immunological tolerance: Forbidden clones allowed in tetraparental mice. Proc. Nat. Acad. Sci Wash. 68:1644-1647 (1971).

Weigle, W.O.: The production of thyroiditis and antibody following injection of unaltered thyroglobulin without adjuvant in rabbits previously stimulated with altered thyroglobulin. J. Exp. Med. 122:1049-1062 (1965).

Weigle, W.O., and R.M. Nakamura: The development of autoimmune thyroiditis in rabbits following injection of aqueous preparations of heterologous thyroglobulins.

J. Immunol. 99:223-231 (1967).

Weiss, D.W., and O. Main: The effect of pre and neonatally injected Diphtheria toxoid on the homologous responsiveness of young guinea pigs. A preliminary report. Immunology. 5:333-339 (1962).

Wilson, D.B., and P.C. Nowell: Quantitative studies on the mixed lymphocyte interaction in rats. IV. Immunologic potentiality of the responding cells. J. Exp. Med. 131:391-407 (1970).

Wilson, D.B., W.K. Silvers, and P.C. Nowell: Quantitative studies on the mixed lymphocyte interaction in rats. II. Relationship of the proliferative response to the immunologic status of the donors. J. Exp. Med. 126:655-665 (1967).

Wright, P.W., I.D. Bernstein, B-Hamilton, and K.E. Hellstrom: In-vitro reactivity in allograft tolerance. Persistence of cell-mediated cytotoxicity and serum blocking activity in highly tolerant rats. Transplant. 19:437-441 (1975).

Wu, S.: Ontogeny of cell-mediated immunity of murine thymocytes and spleen cells. Differentiation. 11:169-174 (1978).